

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
10 January 2002 (10.01.2002)

PCT

(10) International Publication Number
WO 02/02764 A2

- (51) International Patent Classification⁷: **C12N 11/00**
- (21) International Application Number: PCT/US01/20395
- (22) International Filing Date: 26 June 2001 (26.06.2001)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
09/607,716 30 June 2000 (30.06.2000) US
- (63) Related by continuation (CON) or continuation-in-part (CIP) to earlier application:
US 09/607,716 (CON)
Filed on 30 June 2000 (30.06.2000)
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- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,
MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK,
SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA,
ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— without international search report and to be republished
upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guide-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: HIGH MOLECULAR WEIGHT DERIVATIVES OF VITAMIN K-DEPENDENT POLYPEPTIDES

(57) Abstract: Modifications of vitamin K-dependent polypeptides that lead to enhanced protein function on a weight or molar basis and/or increase of protein lifetime in the circulation are described. Both objectives are important for using vitamin K-dependent polypeptides for pro- and anti-coagulation therapies, as well as for other uses in the circulation.

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HIGH MOLECULAR WEIGHT DERIVATIVES OF VITAMIN K-DEPENDENT POLYPEPTIDES

STATEMENT AS TO FEDERALLY-SPONSORED RESEARCH

5 Funding for work described herein was provided in part by the federal government, which may have certain rights in the invention.

TECHNICAL FIELD

10 This invention relates to high molecular weight derivatives of vitamin K-dependent polypeptides, and more particularly to dimerized vitamin K-dependent polypeptides and vitamin K-dependent polypeptides that are linked to PEG polymers.

BACKGROUND

15 Vitamin K-dependent proteins contain 9 to 13 gamma-carboxyglutamic acid residues (Gla) in their amino terminal 45 residues. The Gla residues are produced by enzymes in the liver that utilize vitamin K to carboxylate the side chains of glutamic acid residues in protein precursors. Vitamin K-dependent proteins are involved in a number of biological processes, of which the most well described is blood coagulation (reviewed in Nelsestuen (2000) Vitam. Horm. 58:355-389). Vitamin K-dependent proteins include protein Z, protein S, prothrombin (factor II), factor X, factor IX, protein C, factor VII, Gas6, and matrix GLA protein. Factors VII, IX, X and II function in procoagulation
20 processes while protein C, protein S and protein Z serve in anticoagulation roles. Gas6 is a growth arrest hormone encoded by growth arrest-specific gene 6 (*gas6*) and is related to protein S. See, Manfioletti et al. (1993) Mol. Cell. Biol. 13:4976-4985. Matrix GLA protein normally is found in bone and is critical to prevention of calcification of soft tissues in the circulation. Luo et al. (1997) Nature 386:78-81.

25 The regulation of blood coagulation is a process that presents a number of leading health problems, including both the failure to form blood clots as well as thrombosis, the formation of unwanted blood clots. Agents that prevent unwanted clots are used in many situations and a variety of agents are available. Unfortunately, most current therapies

have undesirable side effects. Orally administered anticoagulants such as Warfarin act by inhibiting the action of vitamin K in the liver, thereby preventing complete carboxylation of glutamic acid residues in the vitamin K-dependent proteins, resulting in a lowered concentration of active proteins in the circulatory system and reduced ability to form
5 clots. Warfarin therapy is complicated by the competitive nature of the drug with its target. Fluctuations of dietary vitamin K can result in an over-dose or under-dose of Warfarin. Fluctuations in coagulation activity are an undesirable outcome of this therapy.

Injected substances such as heparin, including low molecular weight heparin, also are commonly used anticoagulants. Again, these compounds are subject to overdose and
10 must be carefully monitored.

A newer category of anticoagulants includes active-site modified vitamin K-dependent clotting factors such as factor VIIa and IXa. The active sites are blocked by serine protease inhibitors such as chloromethylketone derivatives of amino acids or short peptides. The active site-modified proteins retain the ability to form complexes with their
15 respective cofactors, but are inactive, thereby producing no enzyme activity and preventing complexing of the cofactor with the respective active enzymes. Thus, active-site modified Factor VIIa, denoted factor VIIai, still binds tissue factor, but does not have enzyme activity. Active site-modified proteins appear to have very beneficial anti-coagulant properties with few undesirable side affects. For example, factor VIIai has
20 been shown to lower platelet deposition at the site of surgery, an important indicator of anti-coagulation action. While this can also be accomplished by heparin or other anticoagulants, factor VIIai was unique in that its administration was not accompanied by increased bleeding time or blood loss. See, Harker et al. (1997) Thromb. Haemost. 78:736-741. A similar outcome was reported when factor IXai was administered during
25 surgery. See, Spanier et al. (1998) J. Thorac. Cardiovasc. Surg. 115(5):1179-88. In short, these proteins appear to offer the benefits of anticoagulation therapy without the adverse side effects of other anticoagulants. Active site modified factor Xa is another possible anticoagulant in this group. Its cofactor protein is factor Va. Active site modified activated protein C (APC) may also form an effective inhibitor of coagulation. See,
30 Sorensen et al. (1997) J. Biol. Chem. 272:11863-11868. Active site modified APC binds to factor Va and prevents factor Xa from binding.

A major inhibition to the use of active site-modified vitamin K-dependent clotting factors is cost. Biosynthesis of vitamin K-dependent proteins is dependent on an intact glutamic acid carboxylation system, which is present in a small number of animal cell types. Overproduction of these proteins is severely limited by this enzyme system.

Furthermore, the effective dose of these proteins is high. A common dosage is 1000 µg of VIIai/kg body weight. See, Harker et al. 1997 *supra*. Current cost (April of 2000) of recombinant factor VIIa is about \$0.80 per µg, which severely limits use.

A second problem for several of these proteins is a short lifetime in the circulation system. The situation for factor VIIa illustrates this problem. Factor VII and VIIa have circulation half-times of about 2-4 hours in humans. That is, within 2-4 hours, half of the protein is taken up by other tissues of the body. When factor VIIa is used as a procoagulant to treat certain forms of hemophilia, the standard protocol is to inject VIIa every two hours and at high dosages (45 to 90 µg/kg body weight). See, Hedner et al. (1993) Transfus. Med. Rev. 7:78-83. Thus, use of these proteins as procoagulants or anticoagulants (in the case of factor VIIai) requires that the proteins be administered at frequent intervals and at high dosages.

SUMMARY

The invention is based, in part, on modifications to vitamin K-dependent polypeptides that increase their circulation half-life and in some embodiments, their activity. Both outcomes reduce the amount of protein needed to treat clotting disorders as well as decrease the frequency of administration. As a result, costs associated with treating patients can be reduced, allowing the therapies to be made more widely available to individuals in need of pro- or anti-coagulation therapies.

In one aspect, the invention features an isolated vitamin K-dependent polypeptide linked (e.g., directly or indirectly) to a polyethylene glycol (PEG) polymer. The polypeptide can be selected from the group consisting of factor VII, factor II, protein C, protein S, gas6, and bone matrix Gla protein or can be a protease selected from the group consisting of factor VIIa, factor IIa, and activated protein C. Factor VIIa is a particularly useful protease. The protease can be further linked to an active-site inhibition reagent such as a chloromethylketone derivatized amino acid or peptide. In some embodiments,

the PEG polymer is linked to the protease via the active-site inhibition reagent. Such modified vitamin K-dependant polypeptides can be used in the manufacture of a medicament for procoagulant or anticoagulant therapy.

5 The invention also features an active-site inhibition reagent linked to a PEG polymer. The reagent can be a chloromethylketone derivatized amino acid or peptide or a phosphohalide derivative.

In another aspect, the invention features an anticoagulant agent that includes two polypeptide monomers, wherein at least one of the polypeptide monomers is a vitamin K-dependent polypeptide, and wherein the polypeptide monomers are covalently linked.

10 The polypeptide monomers can be covalently linked via a bi-functional active-site inhibition reagent. The two polypeptide monomers can be the same or different polypeptides. In some embodiments, each of the two polypeptide monomers is a vitamin K-dependent polypeptide, such as a factor VIIa polypeptide, a factor Xa polypeptide, or a factor IXa polypeptide. The bi-functional active-site inhibition reagent can be linked to a

15 PEG polymer. At least one of the polypeptide monomers also can be directly linked to a PEG polymer. Such anticoagulant agents can be used in the manufacture of a medicament for anticoagulant therapy.

The invention also features a bifunctional active-site inhibition reagent that includes two covalently linked active-site inhibitors. At least one of the active-site

20 inhibitors can be linked to a PEG polymer.

A method of directly linking a vitamin K-dependent polypeptide to a PEG polymer also is featured. The method includes incubating the PEG polymer with the vitamin K-dependent polypeptide for a time sufficient to link the PEG polymer to the vitamin K-dependent polypeptide, wherein the PEG polymer is reactive with amino

25 groups or carbohydrate groups on the vitamin K-dependent polypeptide.

In yet another aspect, the invention features a method of indirectly linking a vitamin K-dependent polypeptide to a PEG polymer. The method includes providing a PEG-modified, active-site inhibition reagent, wherein the PEG polymer is reactive with amino groups on the active-site inhibition reagent; and incubating the PEG-modified,

30 active-site inhibition reagent with the vitamin K-dependent polypeptide for a time

sufficient to link the PEG modified, active-site inhibition reagent to the vitamin K-dependent polypeptide.

A method of making an anticoagulant agent also is featured. The method includes incubating a bi-functional active-site inhibition reagent and at least one vitamin K-dependent polypeptide in the presence of phospholipid for a time sufficient to link the bi-functional active-site inhibition reagent and the vitamin K-dependent polypeptide.

In another aspect, the invention features a pharmaceutical composition that includes an isolated vitamin K-dependent polypeptide linked to a PEG polymer and a pharmaceutically acceptable carrier. The polypeptide and PEG polymer can be indirectly linked. The polypeptide can be a protease selected from the group consisting of factor VIIa, factor IXa, factor Xa, factor IIa, and activated protein C. Factors VIIa, IXa, and Xa are particularly useful proteases. The protease can be further linked to an active-site inhibition reagent such as a chloromethylketone derivatized amino acid or peptide. The PEG polymer can be linked to the protease via the active-site inhibition reagent.

The invention also features a pharmaceutical composition that includes an anticoagulant agent and a pharmaceutically acceptable carrier, wherein the anticoagulant agent includes two polypeptide monomers, wherein at least one of the polypeptide monomers is a vitamin K-dependent polypeptide, and wherein the polypeptide monomers are covalently linked. The polypeptide monomers can be covalently linked via a bi-functional active-site inhibition reagent.

In yet another aspect, the invention features a method for evaluating dosage of factor VIIa. The method includes obtaining a biological sample from a patient undergoing factor VIIa therapy; and monitoring clotting time of the biological sample in a device, wherein the device comprises an activator of the contact phase of coagulation and is lacking added phospholipid, wherein a sufficient decrease in clotting time compared to a control sample from the patient before the factor VIIa therapy indicates that an appropriate dosage of factor VIIa has been administered. Factor VIIa therapy can include administering to the patient factor VIIa linked to a PEG polymer.

The invention also features a method for managing anticoagulation therapy in a patient. The method includes administering an acute phase anticoagulant to the patient during the acute phase of coagulation; and administering an active-site inhibited factor

VIIa polypeptide to the patient during the chronic phase of coagulation. The active-site inhibited factor VIIa polypeptide can be linked to a PEG polymer, as described above. The acute phase anticoagulant can be active-site modified factor IXa or active-site modified Xa.

Pharmaceutical compositions that include an active-site inhibited factor VIIa polypeptide and an acute phase anticoagulant also are featured. The active-site inhibited factor VIIa polypeptide can be linked to a PEG polymer, as described above. The PEG polymer can be linked to the active-site inhibited factor VIIa polypeptide via an active-site inhibition reagent.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

Figures 1A-1E are schematics of strategies for modifying vitamin K-dependent polypeptides.

Figure 2 is a graph depicting inhibition of acute coagulation by VIIai-PEG. Titrations of VIIai (open circles), VIIai(PEG-40,000) (solid circles), Q10E32ai (open squares), and Q10E32ai(40,000) (closed squares) are shown. In the inset of Figure 2, VIIai function versus PEG-MW is depicted.

Figure 3 is a graph depicting inhibition of chronic coagulation. Samples include factor VIIai (open circles), VIIai(PEG-40,000) (solid circles), Q10E32ai (open squares), and Q10E32ai(PEG-40,000) (solid squares).

Figures 4A and 4B are graphs depicting activity of dimeric proteases. Figure 4A is a graph of clotting time versus concentration of VIIai (solid circles) and dimeric VIIai (open circles when calculated on the basis of monomer concentration and open squares when calculated on the basis of dimer concentration). Figure 4B is a Hill type plot of the data shown in Figure 4A.

Figure 5 is a Hill type plot of the activity of the VIIai (solid circles) vs. that of the heterodimer, VIIai-Xai (open circles).

Figures 6A and 6B are Hill-type plots depicting activity of inactivated proteases. Figure 6A is a comparison of wild type factor VIIai (solid circles) with monomeric VIIai(P10Q/K32E) (open triangles), dimeric VIIai(P10Q/K32E) containing over 50% dimer (open circles), and VIIai(P10Q/K32E) containing traces of dimer (open squares). Figure 6B is a comparison of wild type VIIai (solid circles) with mutant VIIai(P10Q/K32E) (open squares) and a heterodimer of mutant VIIai(P10Q/K32E)-Xa (open triangles).

Figure 7 is a graph of clotting time (in sec.) versus concentration of dimeric Xai (nM).

Figure 8A and 8B are graphs depicting circulation of VIIai in the mouse. In panel A, analysis of disappearance from zero time is depicted. Factor VIIai concentrations were standardized to the amount present at the first sample (5 to 8 min) and are plotted by a first order decay. In panel B, samples for 5 animals receiving VIIai (open symbols) are shown, as well as 3 animals receiving VIIai(PEG-3400) (Solid symbols). All VIIai levels are standardized to the amount present at the 70-90 minute time point.

Figure 9 is a graph depicting the circulation time for VIIai-PEG of different MW. VIIai levels are shown relative to the amount present at 70-90 minutes. From left to right, the decay curves and first rate constants for circulation turnover are: VIIai, 0.00745/min; VIIai(PEG-3400), 0.00345/min; VIIai(PEG-20,000), 8.25×10^{-4} /min; VIIai(PEG-40,000), 4.28×10^{-4} /min and VIIai(PEG-80,000), 2.26×10^{-4} /min.

Figure 10 is a graph depicting the circulation half-time as a function of PEG. Circulation half-times for PEG derivatives in Figure 9 are plotted vs. PEG-MW. The intercept is at 93 min, the half-time of VIIai without PEG.

Figures 11A and 11B are graphs depicting the circulation time for factor VIIai proteins. Figure 11A shows results for three animals that were administered VIIai (solid circles, open diamonds, and inverted triangles), one animal that was administered dimeric VIIai (open squares), and two animals that were administered VIIai-PEG-3.4k (solid triangles and solid diamonds). Figure 11B provides the turnover of randomly modified VIIai. Two lines are drawn for the first three and the last three data points. VIIai is shown for reference (solid circles).

Figures 12A and 12B are graphs of clotting time (sec) versus factor VIIa concentration. In Figure 12A, factor VIIa and calcium were added to anticoagulated blood of two hemophilia patients (open circles and solid circles), just before assaying. Titration with the factor VIIa mutant, P10Q/K32E (solid squares), is shown for the same patient who received wild-type VIIa (shown in solid circles). Figure 12B provides results for blood from a normal individual that had been treated with anti-VIII antibody to create severe VIII deficiency. Experiments are shown for wild type VIIa (open circles), the VIIa-P10Q/K32E mutant (open squares), PEG-derivatized wild type VIIa (solid diamonds), and PEG-derivatized VIIa-P10Q/K32E (solid circles).

Figures 13A and 13B are graphs comparing acute versus chronic anticoagulation. Figure 13A depicts factor VIIai inhibition in acute coagulation (open circles) and chronic coagulation (solid circles) and Q10E32ai inhibition of acute coagulation (open squares) and chronic coagulation (solid squares). Figure 13B is a time course for inhibition by factor VIIa, the conversion from acute to chronic anticoagulation. Results are for 10 nM factor VIIai-WT (open squares), 0.4 nM Q10E32ai (open circles), 3.8 nM Q10E32ai (solid squares), 4 nM factor VIIa-WT (open triangles), and 0.17 nM Q10E32ai (solid circles).

DETAILED DESCRIPTION

Vitamin K-dependent polypeptides are a group of proteins that utilize vitamin K in their biosynthetic pathways to carboxylate the side chains of glutamic acid residues in protein precursors. The GLA domain contains 9-13 γ -carboxyglutamic acid residues in the N-terminal region of the polypeptide, typically from amino acid 1 to about amino acid 45. Protein Z, protein S, factor X, factor II (prothrombin), factor IX, protein C, factor

VII, Gas6, and bone matrix GLA protein are examples of vitamin K-dependent polypeptides that are useful in the invention. Furthermore, useful vitamin K-dependent polypeptides can be wild-type or can contain mutations. Particularly useful factor VII and protein C mutations are described in Shah et al. (1998) Proc. Natl. Acad. Sci. USA 95:4229-4234 and Shen et al. (1998) J. Biol. Chem. 273:31086-3101, respectively, in which enhancements in protein function were reported. Also see U.S. Patent No. 6,017,882 and WO 00/66753. For example, factor VII or active site modified activated factor VII (factor VIIai) containing a glutamine at amino acid 10 and a glutamic acid at amino acid 32 (designated Q10E32 or P10Q/K32E) can be used. Many clotting factors, including factors VII, IX, X, and prothrombin, are zymogens, i.e., inactive proenzymes, and are converted during coagulation to active serine proteases. Active proteases are designated with an "a", e.g. factor VIIa.

Figure 1 provides a general description for making derivatives of vitamin K-dependent polypeptides of the invention. The polypeptides presented in Figure 1 are described as proteases. It should be noted, however, that many of the reactions, including reactions A2, A2' and E5, can be used with any vitamin K-dependent polypeptide.

Vitamin K-dependent polypeptides linked to PEG polymers

The invention features isolated vitamin K-dependent polypeptides linked to polyethylene glycol (PEG) polymers. An "isolated polypeptide" has been separated from cellular components that naturally accompany it. Typically, the polypeptide is isolated when it is at least 60% (e.g., 70%, 80%, 90%, or 95%), by weight, free from proteins and naturally-occurring organic molecules that are naturally associated with it. As used herein, the term "polypeptide" is any chain of at least five amino acids that retains the ability to bind cofactors or membranes. Amino acids have been designated herein by standard three letter and one-letter abbreviations.

Derivatization of amino groups on the surface of proteins (m in Figure 1A) with PEG-containing compounds can extend the circulation time of some proteins and reduce their potential antigenic properties. See, Beauchamp et al. (1983) Anal. Biochem. 131(1):25-33. In general, the greatest impact on circulation time has been with longer chain PEG polymers, with a molecular weight up to about 15,000. PEG modified

proteins typically are low molecular weight proteins that are cleared from the circulation by pinocytosis in the kidney, such as a Fv fragment of an antibody molecule, and proteins that act on low molecular weight substrates, such as superoxide dismutase, asparaginase, and hemoglobin. Lee et al. (1999) Bioconjugate. Chem. 10:973-981; and Shearwater Polymers, Inc. (2000) Catalog, p. 41, Huntsville, AL. The increase of molecular weight by addition of PEG to low molecular weight proteins is thought to enhance circulation lifetime by eliminating a specific clearance process.

PEG-derivatization of proteins also can reduce protein antigenicity. Thus, PEG derivatization can reduce or prevent antibody production to a foreign protein. Without being bound by a particular mechanism, a 'polymer cloud' of PEG may diffuse at the surface of the protein, obliterating the surface for the macromolecular recognition system of antibody production. Shearwater Polymers, Inc. (2000) Catalog, p 11. Based on this mechanism, PEG modification may hinder interaction of the modified protein with other macromolecules while allowing small molecule diffusion near the protein surface such that substrate access to the active site is virtually unchanged. Thus, activity of proteins with low molecular weight substrates, such as superoxide dismutase, is not altered, whereas binding between an antibody and antigen may be diminished with multiple PEG derivatizations. See, Lee et al. (1999) Bioconjugate. Chem. 10:973-981.

Based on this, factor VII and other vitamin K-dependent polypeptides such as factors IX, X, and II that interact with cofactor proteins, membrane surfaces, or both, appear to be unlikely targets for PEG modification. For example, factor VIIa must interact with tissue factor, a macromolecular cell surface receptor that includes a large area of the factor VIIa surface, and with a membrane surface. See, Banner et al. (1996) Nature 380:41-46. The presence of a polymer cloud over the factor VIIa surface would be expected to interfere with both of these critical interactions. In addition, the vitamin K-dependent polypeptides occur naturally in the plasma and have molecular weights that are high enough to avoid rapid removal through the kidney. As described herein, however, vitamin K-dependent polypeptides that are linked to PEG have an increased lifetime in the circulation, with little or no impact on activity. PEG modified proteins may have greater stability during storage and lyophilization than unmodified polypeptides.

The term "linked" is used herein to include 1) both direct, covalent links and indirect links (i.e., through an intermediate molecule) of a PEG polymer to a polypeptide; 2) covalent coupling of a PEG polymer to an intermediate molecule such as an active-site inhibition reagent; 3) covalent coupling of an active-site inhibition reagent to a protease; and 4) direct, covalent links and indirect links (i.e., through an intermediate molecule) of polypeptide monomers to each other. In some embodiments, the PEG polymer is directly and indirectly linked to the vitamin K-dependent polypeptide. Suitable PEG polymers can vary in length and valency with respect to the number of PEG chains per reactive site, n or m in Figure 1. Typically, PEG polymers contain an activated ester (-CO-Y) and can react with protein amino groups to form a covalent linkage. Many standard leaving groups (Y) are known and are available commercially. Non-limiting examples of leaving groups include p-nitrophenol and succinimidyl propionate (SPA). In addition to activated esters, other standard approaches exist for crosslinking a reagent to amino groups on the protein surface, including, for example, aldehyde-containing PEG molecules. The aldehyde forms a Schiff's base with amino groups on the protein. The Schiff's base is selectively reduced with sodium cyanoborohydride in a well-described reaction. This chemistry is available commercially. See, Shearwater Polymers, Inc. (2000) Catalog, p. 20. Another process for attaching groups such as PEG to glycoproteins uses periodate to oxidize carbohydrates to aldehydes, followed by addition of hydrazide derivatives of the group to be attached. The hydrazide reacts with the aldehydes to produce a stable link. Hydrazide derivatives of PEG polymers are available commercially, for example, from Shearwater Polymers, Inc. and appropriate reaction conditions are described in the Shearwater Polymers, Inc. catalog (2000, p. 34). Many of the vitamin K-dependent polypeptides are glycoproteins and therefore subject to this chemistry.

PEG polymers can be directly linked to vitamin K-dependent polypeptides, including active-site modified proteases, by randomly reacting the PEG polymer with protein amino moieties. Reactions in Figure 1A produce heterogeneous products (1 to m derivatives at random locations) as a typical polypeptide may have 20 amino groups on its surface and there is little basis for chemical selectivity. As described herein, a population of factor VIIaI molecules with randomly linked PEG had circulation lifetimes that were 20 times greater than those of the wild type protein, although activity was slightly less.

While products II and III in Figure 1 are heterogeneous with respect to both the number of PEG attachments and location of the PEG on the polypeptides, these preparations have potential value to therapy. Standardization of reaction conditions can create preparations with consistent properties that may be beneficial for both pro- and anticoagulation therapy.

Reactions B1 and B2 of Figure 1B illustrate methods used to indirectly derivatize polypeptides by attachment of the PEG polymer through the active site of the protease. In general, a PEG polymer and an active-site inhibition reagent (described below) can be covalently linked to form a PEG-modified active-site inhibition reagent before reacting with an activated vitamin K-dependent polypeptide, such as factor VIIa, IXa, Xa, or IIa. Since the active-site-directed inhibitor has a single amino group, only one product (compound IV) is generated from reaction B1. Unreacted active-site-directed reagent can be removed by dialysis or gel filtration chromatography. The PEG polymer has a high molecular weight and these simple procedures allow quantitative separation from unreacted reagent. The active ester on the PEG moiety ($-\text{CO}-\text{Y}$) is slowly hydrolyzed by water, and incubation for a period of time can remove excess reactive reagent. Thus, it may not be necessary to remove any excess PEG before proceeding with step B2. Once all of the activated ester is destroyed by water hydrolysis, step B2 can be initiated.

In step B2, the PEG-modified, active site-directed reagent reacts with the protease, with negligible side reactions, to produce a single entity. Excess PEG, in any form, can be removed by ion exchange chromatography on a material such as DEAE-Sephadex, which is a standard step in the purification of vitamin K-dependent polypeptides. These polypeptides bind tightly to anion exchange material at low salt (e. g. 0.1 M NaCl, pH 7.0) and are eluted by high salt (e. g. 0.5 M NaCl). As unreacted PEG polymers have little charge (the active site-directed reagent is cationic, if it is present) and do not bind to an anion exchange resin, the polymers can be removed by washing the column with low salt buffer before eluting with high salt. The reactions described in Figure 1B can use a range of leaving groups (Y) or other strategies for derivatizing amino groups as outlined above. Purification of the final product may not be necessary as unreacted PEG polymers are biologically inert and active-site inhibition reagents may not pose adverse effects.

The PEG derivatives of blood clotting proteases described herein provide an enormous advantage for use in either procoagulant (e.g. factor VIIa or IXa) or anticoagulant (Factor VIIai, IXai, Xai or IIai) therapy. The longer circulation time not only decreases the amounts needed for anticoagulation over time, but also greatly diminishes the frequency with which the anticoagulants have to be administered by injection. Circulation lifetime of PEG modified polypeptides was determined by the size of the PEG polymer attached. In particular, PEG modification at the active site of wild type factor VIIai prolonged circulation time in the mouse by the relationship: halftime (in minutes) = $94 + 37 * (\text{molecular weight of PEG in Daltons})$. Factor VIIai-PEG (with PEG=40,000) had a circulation halftime of 28 hours, an 18-fold increase over unmodified factor VIIai, and retained 30% of its anticoagulant function. This relationship extended to a mutant of VIIai (Q10E32ai) that had 25-fold higher function than WT-VIIai. Overall, the Q10E32ai-PEG (PEG=40,000) derivative had 8-fold higher function and 18-fold longer circulation lifetime than WT-VIIai, a 140-fold enhancement of efficacy. High retention of activity did not extend to factors Xai or IXai, which lost almost all activity upon PEG modification. Factor VIIa that was modified with PEG on lysine side chains also lost most function. The magnitude of impact of PEG on circulation time of factor VIIai was very surprising, given that this protein is a naturally occurring plasma protein and that it is not subject to removal as a low molecular weight peptide.

In some embodiments, PEG polymers having more than one reactive group (e.g., 2-4) per polymer can be used to modify the vitamin K-dependent polypeptide. PEG polymers with more than one reactive group are commercially available from, for example, Shearwater Polymers, Inc. PEG polymers with other combinations of reactive groups can be generated by chemical synthesis using known chemistry. The reactions in Figure 1C utilize multivalency of reactive groups on the PEG polymer to generate multivalency with respect to active-site modified proteases. Quantitative yields in reaction C1 are difficult to achieve due to water hydrolysis of the active ester. In addition, with, e.g., four reactive groups per polymer, one can expect PEG products containing 0, 1, 2, 3, and 4 active site-directed inhibitors. Purification of each product is possible, although more complex than purification of compound IV in Figure 1B. Since the active-site-directed inhibitor typically contains the side chain of an arginine residue,

each active-site inhibition reagent that is present adds a charge and the products can be separated by ion exchange chromatography. Each peptide of the active-site inhibition reagent also can enhance affinity of the product for reverse phase chromatography on a C18 column.

5

Active site inhibition reagents

Chloromethylketones or organic phosphohalides such as diisopropylfluorophosphate (DIFP), as well as other known chemistries, can be used to modify the active-site of proteases that are vitamin K-dependent polypeptides.

10

Chloromethylketones can be single amino acids such as tosyllysylchloromethylketone (TLCK) or short peptides and are commercially available.

Phenylalanylprolylarginylchloromethylketone (FPRck, also known as PPACK) is available from Calbiochem, San Diego, CA and is described by the manufacturer as a good inhibitor of thrombin and factor VIIa, among other enzymes.

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Phenylalanylphenylalanylarginylchloromethylketone (FFRck, also known as PPACK II) is available from Calbiochem and is another example of a commonly available peptide. Glutamylglycylarginylchloromethylketone (EGRck) can be used with a derivative at its amino terminus, such as the fluorescent derivative, Dansyl-EGRck (DEGRck), and also is available from Calbiochem.

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Reactions A1 and A1' of Figure 1 depict reaction of a chloromethylketone derivative (ck) of an active site-directed peptide or amino acid (H_2N-XXX) with the active site of a blood clotting protease (e.g., Factor VIIa, IXa, Xa, thrombin or factor IIa) to create an inactive enzyme (VIIai, Xai, IXai, IIai).

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Oligomers of Vitamin K-dependent polypeptides

The invention also features anticoagulant agents that include oligomers of vitamin K-dependent polypeptides. In general, such anticoagulant agents include at least two polypeptide monomers that are covalently cross-linked. At least one of the polypeptide monomers is a vitamin K-dependent polypeptide, while the second polypeptide monomer can be the same or a different vitamin K-dependent polypeptide, a protease (e.g., trypsin), or a polypeptide that contains a membrane-binding site. Annexin polypeptides are non-limiting examples of polypeptides that contain a membrane binding site. Figure 1D

30

provides reactions for producing a homodimer of active site-modified blood clotting proteases. Heterodimers can be produced in a similar manner. Homodimer refers to a dimer containing two identical polypeptides, while heterodimer refers to a dimer containing two different polypeptides. Typically, a bifunctional reagent is reacted with an active-site inhibition reagent (reaction D1 and D1'), and the resulting bifunctional active-site inhibition reagent containing two active-site inhibitors is isolated. The monovalent product also can be isolated and may be useful in some embodiments.

Diethylenetriaminepentacetic acid anhydride (DTPA) is an example of bifunctional reagent that can be used. Many other bifunctional reagents that react with amines are available commercially. For example, the Pierce Chemical Co. (Rockford, IL) has 49 bifunctional reagents available that react with amines.

HPLC chromatography can be used to separate univalent products from divalent products. The bifunctional active-site inhibition reagent can be used to create various homodimers and heterodimers of blood clotting proteases, such as compound VI of Figure 1D. Addition of phospholipid vesicles to the reaction can increase dimer yield. In some cases, heterodimers may be more effective *in vivo* due to simultaneous inhibition of several steps of coagulation. For example, a factor Xai-factor IXai dimer can inhibit factors VIIa and Va at the same time, while a VIIai-Xai dimer can inhibit tissue factor and factor Va at the same time. Homo- and heterodimers can be purified by standard methods, including, for example, gel filtration chromatography.

As described herein, dimers of wild type factor VIIai enhanced protein-cofactor-membrane binding by about 16-fold, which is much less than the theoretical increase of 10^6 to 10^{11} -fold. The heterodimer of VIIa and factor Xa produced a 25-fold increase in competitive binding to tissue factor-membrane, much less than the theoretical level of about 10^7 -fold. Factor Xa has a K_d for membrane association of about 10^{-7} . See, McDonald et al. (1997) *Biochemistry* 36:5120-5127. Dimerization of a mutant of factor VIIa (P10Q/K32E), which has higher affinity for the membrane than wild type factor VIIa, did not result in detectable improvement of binding affinity. See, Shah et al., *supra*, for a description of the mutant. This indicates that minor changes in the membrane-binding site altered the ability to participate in simultaneous binding. The heterodimer of mutant VIIa and factor Xa actually had reduced affinity for tissue factor-membrane than

did monomeric mutant VIIai. While a 16-35-fold improvement in affinity can have a large impact on therapeutic value of a drug, this range is a very small part of the total free energy of the multiple binding events.

5 *Dimerized, active-site modified, PEG-linked vitamin K-dependent polypeptides*

As described above, PEG polymers can be introduced to random surface amine groups or to the active site of a protease to enhance its circulation time and active site-modified enzymes can be dimerized to enhance function. Such protein modification strategies can be combined to provide dimeric, active site-modified proteases that are
10 linked to PEG polymers, resulting in polypeptides with up to several hundred-fold enhancement of function over wild type, monomeric, active site-modified proteases. Well-known and established chemistries can be used to produce such modified polypeptides, as illustrated in Figure 1E. Reactions E1, E2, E1' and E2' are continuations of the DTPA dimerization shown in Figure 1D and combine dimer formation and
15 introduction of PEG into the same molecule. Both DTPA derivatives of the active site-directed inhibitor contain carboxyl groups but no amino groups. The DTPA derivatives can be crosslinked to appropriate amines by well-known reactions such as that of carbodiimide, a reagent that is commonly used for amide and peptide bond synthesis. Both the monomeric and dimeric inhibitor products can be modified by these reactions.

20 When univalent PEG is used as the source of amino groups ($m=1$ in Figure 1E), a range of products can be produced that differ in the number of PEG polymers attached to the active-site-directed reagent. For example, a divalent active site-directed intermediate can produce a modified polypeptide that has the longer circulation time provided by PEG and the higher activity of dimeric VIIai. An intermediate with a single active-site-directed inhibition reagent can produce an inhibitor with the advantage of multiple PEG
25 polymers, giving enhanced circulation lifetime.

If multivalent PEG ($m>1$, Figure 1E) is used as the source of amino groups, complex products can be produced such as an oligomer of monomers (reaction E2') or an oligomer of dimers (reaction E2). Products generated by reactions E1 and E2 may be
30 heterogeneous due to difficulty in obtaining quantitative or strictly controlled reaction with carbodiimide. Nevertheless, heterogeneity does not prevent formation of useful

products. Since multivalency occurs on both the amino group and the carboxyl group, some products may be extremely large.

Reaction E3 outlines a strategy for introducing multiple PEG polymers to the active site of a vitamin K-dependent protease. This can be accomplished, for example, by using an active-site inhibition reagent that includes additional amino groups. The example shown in reaction E3 is a short polylysine chain (K_7). The PEG-linked product can be coupled to factor VIIa by reaction B2.

Crosslinkers with longer spacer arms can be produced by reaction E4, using the reactions described in Figure 1D, but with different active-site inhibition reagents. Use of linker arms of different length is well-established in crosslinking technology. Several crosslinking agents with different lengths (e.g. linker arms of 2 to >1000 atoms) are available from commercial sources such as the Pierce Chemical Co. and the Sigma Chemical Co. For example, elongated, bifunctional active-site inhibition reagents with more than three amino acids can be produced and the remaining reactions carried out as in Figure 1B. The advantage of reaction E4 may be the ability to utilize a wider range of proteins. Using a longer linker arm, generated by the simple modification shown in reaction E4 (or numerous other strategies), may result in dimers with enhanced activity.

Reaction E5 shows another benefit of dimeric proteins. That is, random derivatization of a dimer with one PEG polymer leaves one polypeptide monomer without modification. That is, addition of one PEG polymer to a dimeric polypeptide leaves one polypeptide monomer completely underivatized and free to bind to its receptor or cofactor protein. In fact, by random attachment, fifty percent of a dimer population that has an average of two PEG polymers attached still will have one polypeptide monomer that is underivatized. The use of dimeric proteins may allow very high order PEG substitution with retention of substantial activity, as randomly derivatized monomeric factor VIIa retained function to the level of at least one PEG per molecule.

Assay for factor VIIa therapy

The invention also provides an assay for factor VIIa therapy that can detect individual variation in sensitivity to VIIa and help target dosage levels for individual patients. No assay is presently available to detect therapeutic response to factor VIIa

administration in an individual hemophilia patient. Assays for hemophilia A and B, its severity and the level of correction by infusion of VIII or IX clotting factors are normally conducted with a plasma clotting assay known as the APTT time. This assay depends on activation of the intrinsic blood clotting cascade (factor XII) by agents such as Kaolin or Ellagic acid. Upon addition of calcium, XIIa activates XI, which activates IX, and coagulation occurs. This assay therefore depends on factors IX and VIII levels in the plasma and is sensitive to both hemophilia A and B. A problem for factor VIIa therapy is that its mechanism of action may differ from the normal cascade. That is, factor VIIa can bind to phospholipid surfaces and activate factor X directly, without use of tissue factor, factor IX, or factor VIII. See, Monroe et al. (1997) Brit. J. Haemat. 99:542-547. In the absence of tissue factor, factor VIIa is a relatively poor enzyme. In addition, factor VIIa has relatively low affinity for a membrane.

Assays that depend on externally added phospholipid do not represent the true procoagulant status of patients undergoing factor VIIa therapy, as phospholipid content influences the outcome of the assay. For example, at high phospholipid concentration, wild type factor VIIa and a mutant factor VIIa protein (P10Q/K32E) have nearly identical activities, while at low phospholipid concentration, an 8-fold difference is observed between the wild type and mutant protein.

As described herein, clotting time can be more accurately monitored by using an assay containing biological membranes rather than phospholipids. Since individuals will vary with respect to their cellular activity, the assay should use the cells of the patient undergoing therapy, at the time at which therapy is administered. Thus, the invention features a method for evaluating dosage of factor VIIa, including PEG-modified factor VIIa. Biological samples such as whole blood are obtained from a patient before and after factor VIIa administration and clotting time is monitored. Typically, a device such as the HEMOCHRON® Jr. from International Technidyne or a similar instrument is used to facilitate the measurement. Such devices contain optical detectors that monitor coagulation time by detecting the speed at which the blood moves between two of the optical detectors. Other devices use resistance to mechanical agitation such as stirring or flow under pressure to detect clotting time. The activated clotting time (ACT) assay and specific instrumentation such as the HEMOCHRON® Jr. microcoagulation apparatus

typically are devoted to monitoring anticoagulation therapy during surgery or other procedures. The results herein, however, indicate that the ACT can provide the necessary conditions required to monitor procoagulation response in hemophilia patients receiving factor VIIa.

5 The biological sample from the patient undergoing factor VIIa therapy is placed in a cuvette or similar container that includes an activator of the contact phase of coagulation and that is lacking added phospholipid. Non-limiting examples of activators of the coagulation phase include Celite, kaolin and ellagic acid. The biological sample and the cuvette contain all necessary components for the reaction. Thus, the cuvette
10 containing the biological sample is placed in the device and clotting time is measured. The clotting times of samples before and after factor VIIa therapy are compared to determine if clotting time has significantly decreased. If a sufficient decrease in clotting time is observed after factor VIIa administration, a sufficient dosage of factor VIIa has been administered. A sufficient decrease in clotting time refers to the restoration of
15 hemostasis in the patient. If clotting time has not sufficiently improved, factor VIIa dosage can be modified appropriately.

 Alternatively, titration curves, similar to those shown in Figure 6, may be constructed before administration of VIIa to an individual. Individual response to factor VIIa is relatively constant over time. Therefore, prospective candidates for factor VIIa
20 therapy can have a titration curve performed ahead of time and used to design therapy when needed.

Acute and Chronic Phases of Coagulation

 The invention also provides a method for managing anticoagulant therapy in a
25 patient. The method includes administering an acute phase anticoagulant to a patient during acute coagulation and administering a factor VIIai polypeptide to the patient during chronic coagulation. "Chronic" anticoagulation refers to equilibrium conditions and can occur when factor VIIa and VIIai are incubated with tissue factor for a time sufficient to reach equilibrium before coagulation is initiated. The assays of Figures 3-6
30 are examples of assays performed under chronic anticoagulation conditions. In contrast, "acute" anticoagulation refers to all components related to coagulation and

anticoagulation being initiated simultaneously. The assay described in Figures 2 and 5, which required Xai to inhibit coagulation, exemplifies acute coagulation. That is, the receptor for Xai, factor Va, is not present until coagulation begins.

Most anticoagulants target materials that are not present until coagulation has begun and therefore act in an 'acute' manner. For example, heparin stimulates antithrombin III inhibition of thrombin; thrombin is not present until coagulation has been initiated. A large number of active site inhibitors of clotting enzymes such as thrombin, Xa, IXa and VIIa all act on a coagulation process that has been initiated. The receptors for factors IXai and Xai (factors VIIa and Va, respectively) are not present until coagulation has begun. Thus, factor VIIai may provide a unique form of anticoagulation that is referred to herein as 'chronic' anticoagulation.

A chronic anticoagulation state can be reached when tissue factor is exposed to the circulation for a prolonged period of time, initiating continual, but very limited, coagulation events. This condition may exist for some time before the tissue factor is removed from the circulation, allowing factor VIIai to reach binding equilibrium and creating 'chronic' anticoagulation. Acute coagulation occurs when the endothelium is damaged, e.g., during angioplasty. After this initial phase, however, chronic coagulation conditions may exist when tissue factor is present for a prolonged period of time. Administration of factor VIIai, and especially factor VIIai with high affinity for tissue factor-membrane, such as dimeric VIIai and mutants with high membrane affinity, during this chronic phase is an effective method of anticoagulation treatment. Although VIIai can prevent acute coagulation, higher dosages are needed. Thus, for many situations such as catheterization, angioplasty, and surgery, effective anticoagulation therapy may be to use a combination of anticoagulants, a highly effective inhibitor of acute coagulation during the procedure that generates tissue injury (such as endothelial cell damage during angioplasty) and factor VIIai to prevent the chronic coagulation that follows. Treatment with VIIai might require several days to provide continual protection until healing is complete and tissue factor is entirely removed from the circulation. Using PEG-derivatized and/or dimerized proteins (e.g., VIIai, Xai, IXai) as acute anticoagulants or chronic anticoagulants during anticoagulation therapy can reduce dosages and reduce the frequency of administration.

Nucleic Acids encoding vitamin K-dependent polypeptides

Isolated nucleic acid molecules encoding vitamin K-dependent polypeptides can be produced by standard techniques. As used herein, "isolated" refers to a sequence corresponding to part or all of a gene encoding a vitamin K-dependent polypeptide, but
5 free of sequences that normally flank one or both sides of the wild-type gene in a mammalian genome. An isolated polynucleotide can be, for example, a recombinant DNA molecule, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, isolated polynucleotides include, without limitation, a DNA that exists as a
10 separate molecule (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated polynucleotide can include a
15 recombinant DNA molecule that is part of a hybrid or fusion polynucleotide.

It will be apparent to those of skill in the art that a polynucleotide existing among hundreds to millions of other polynucleotides within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest, is not to be considered an isolated polynucleotide.

20 Isolated nucleic acid molecules are at least about 14 nucleotides in length. For example, the nucleic acid molecule can be about 14 to 20, 20-50, 50-100, or greater than 150 nucleotides in length. In some embodiments, the isolated nucleic acid molecules encode a full-length vitamin K-dependent polypeptide. Nucleic acid molecules can be DNA or RNA, linear or circular, and in sense or antisense orientation.

25 To obtain mutant vitamin K-dependent polypeptides, specific point changes can be introduced into the nucleic acid sequences encoding wild-type vitamin K-dependent polypeptides by, for example, oligonucleotide-directed mutagenesis. In this method, a desired change is incorporated into an oligonucleotide, which then is hybridized to the wild-type nucleic acid. The oligonucleotide is extended with a DNA polymerase,
30 creating a heteroduplex that contains a mismatch at the introduced point change, and a single-stranded nick at the 5' end, which is sealed by a DNA ligase. The mismatch is

repaired upon transformation of *E. coli* or other appropriate organism, and the gene encoding the mutant vitamin K-dependent polypeptide can be re-isolated from *E. coli* or other appropriate organism. Kits for introducing site-directed mutations can be purchased commercially. For example, Muta-Gene™ in-vitro mutagenesis kits can be purchased
5 from Bio-Rad Laboratories, Inc. (Hercules, CA).

Polymerase chain reaction (PCR) techniques also can be used to introduce mutations. See, for example, Vallette et al., Nucleic Acids Res., 1989, 17(2):723-733. PCR refers to a procedure or technique in which target nucleic acids are amplified. Sequence information from the ends of the region of interest or beyond typically is
10 employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified, whereas for introduction of mutations, oligonucleotides that incorporate the desired change are used to amplify the nucleic acid sequence of interest. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers
15 are typically 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length. General PCR techniques are described, for example in PCR Primer: A Laboratory Manual, Ed. by Dieffenbach, C. and Dveksler, G., Cold Spring Harbor Laboratory Press, 1995.

Nucleic acids encoding vitamin K-dependent polypeptides also can be produced
20 by chemical synthesis, either as a single nucleic acid molecule or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to
25 extend the oligonucleotides, resulting in a double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector.

Production of modified vitamin K-dependent polypeptides

Isolated vitamin K-dependent polypeptides are commercially available from, for example, Novo Nordisk (Princeton, NJ). Vitamin K-dependent polypeptides can be
30 produced in transgenic animals or by cell culture. Preferably, the transgenic animal or

eukaryotic host can carboxylate the glutamic acid residues of the vitamin K-dependent polypeptide. To produce vitamin K-dependent polypeptides by cell culture, a nucleic acid encoding the polypeptide is ligated into a nucleic acid construct such as an expression vector, and eukaryotic host cells are transformed with the expression vector. In general, nucleic acid constructs include a regulatory sequence operably linked to a nucleic acid sequence encoding a vitamin K-dependent polypeptide. Regulatory sequences do not typically encode a gene product, but instead affect the expression of the nucleic acid sequence. As used herein, "operably linked" refers to connection of the regulatory sequences to the nucleic acid sequence in such a way as to permit expression of the nucleic acid sequence. Regulatory elements can include, for example, promoter sequences, enhancer sequences, response elements, or inducible elements.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express vitamin K-dependent polypeptides. A nucleic acid encoding vitamin K-dependent polypeptide can be cloned into, for example, a baculoviral vector such as pBlueBac (Invitrogen, San Diego, CA) and then used to co-transfect insect cells such as *Spodoptera frugiperda* (Sf9) cells with wild-type DNA from *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV). Recombinant viruses producing the vitamin K-dependent polypeptides can be identified by standard methodology. Alternatively, a nucleic acid encoding a vitamin K-dependent polypeptide can be introduced into a SV40, retroviral, or vaccinia based viral vector and used to infect suitable host cells.

Mammalian cell lines that stably express vitamin K-dependent polypeptides can be produced by using expression vectors with the appropriate control elements and a selectable marker. For example, the eukaryotic expression vector pCDNA3.1+ (Invitrogen, San Diego, CA) is suitable for expression of modified vitamin K-dependent polypeptides in, for example, COS cells, HEK293 cells, or baby hamster kidney cells. Following introduction of the expression vector by electroporation, DEAE dextran-, calcium phosphate-, liposome-mediated transfection, or other suitable method, stable cell lines can be selected. Alternatively, transiently transfected cell lines are used to produce vitamin K-dependent polypeptides. Vitamin K-dependent polypeptides also can be transcribed and translated *in vitro* using wheat germ extract or rabbit reticulocyte lysate.

Vitamin K-dependent polypeptides can be purified from conditioned cell medium by applying the medium to an immunoaffinity column. For example, an antibody having specific binding affinity for Factor VII can be used to purify modified Factor VII. Alternatively, concanavalin A (Con A) chromatography and anion-exchange chromatography (e.g., DEAE) can be used in conjunction with affinity chromatography to purify factor VII. Calcium dependent or independent monoclonal antibodies that have specific binding affinity for factor VII can be used in the purification of Factor VII.

Vitamin K-dependent polypeptides such as protein C can be purified by anion-exchange chromatography, followed by immunoaffinity chromatography using an antibody having specific binding affinity for protein C.

Vitamin K-dependent polypeptides also can be chemically synthesized using standard techniques. See, Muir, T.W. and Kent, S.B., Curr. Opin. Biotechnol., 1993, 4(4):420-427, for a review of protein synthesis techniques.

Pharmaceutical Compositions

The invention also features pharmaceutical compositions including a pharmaceutically acceptable carrier and an isolated vitamin K-dependent polypeptide linked to a PEG polymer or an anticoagulant agent that includes at least two polypeptide monomers (described above). The pharmaceutical composition also can include an acute anticoagulant and/or an active-site inhibited factor VIIa polypeptide. The modified vitamin K-dependent polypeptide (e.g., PEG-linked and/or dimerized) is present in an amount effective to alter clot formation in a mammal. Useful vitamin K-dependent polypeptides of the pharmaceutical compositions can include, without limitation, PEG-linked APC, factor VIIai, factor IXai, factor Xai, or factor IIai, as discussed above. Pharmaceutical compositions also can include an additional anticoagulant agent such as aspirin, warfarin, or heparin.

The concentration of a modified vitamin K-dependent polypeptide effective to alter clot formation in a mammal may vary, depending on a number of factors, including the preferred dosage of the compound to be administered, the chemical characteristics of the compounds employed, the formulation of the compound excipients and the route of administration. The optimal dosage of a pharmaceutical composition to be administered

may also depend on such variables as the overall health status of the particular patient and the relative biological efficacy of the compound selected. These pharmaceutical compositions may be used to regulate coagulation *in vivo*. For example, the compositions may be used generally for the treatment of thrombosis.

5 Vitamin K-dependent polypeptides that are linked to a PEG polymer or anticoagulant agents described above may be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable non-toxic excipients or carriers. Such compounds and compositions may be prepared for parenteral administration, particularly in the form of liquid solutions or suspensions in aqueous
10 physiological buffer solutions; for oral administration, particularly in the form of tablets or capsules; or for intranasal administration, particularly in the form of powders, nasal drops, or aerosols. Compositions for other routes of administration may be prepared as desired using standard methods.

Formulations for parenteral administration may contain as common excipients
15 sterile water or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, hydrogenated naphthalenes, and the like. In particular, biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxethylene-polyoxypropylene copolymers are examples of excipients for controlling the release of a compound of the invention *in vivo*. Other suitable parenteral delivery systems include
20 ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation administration may contain excipients such as lactose, if desired. Inhalation formulations may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or they may be oily solutions for administration in the form of nasal drops. If desired, the compounds
25 can be formulated as gels to be applied intranasally. Formulations for parenteral administration may also include glycocholate for buccal administration.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

In all examples shown below, the proteins used are highly purified. For all reactions, with factor VII, commercial factor VIIa was first purified by ion exchange chromatography. The protein (1 to 2 mg) was applied to a column of DEAE-Sephadex (0.5 by 3 cm) and washed with at least 10 column volumes of buffer (0.05 M Tris, pH 7.5). Protein then was eluted with buffer containing 0.5 M NaCl. The resulting factor VIIa was dialyzed to the desired buffer. This procedure removed stabilizing agents in the commercial VIIa that interfered with subsequent modifications. Wild type VIIa or factor VIIa refer to a recombinant protein sold by NOVO Nordisk Company (Princeton, NJ). Factor Xa refers to a purified protein from Enzyme Research Labs (West Bend, IN). Factor IXa refers to a purified protein from Calbiochem (La Jolla, CA).

Example 1 - Synthesis of PEG-linked, active site-directed inhibitor of factor VIIa and other proteases: The chloromethylketone-derivatized peptides FFR and FPR (FFRck and FPRck, respectively, available from Calbiochem or Bachem), were linked to PEG polymers. Commercially available PEG polymers were used that contained reactive groups able to derivatize free amino groups. One PEG polymer contained an activated ester based on a para-nitrophenol leaving group (polyoxyethylene bis para-nitrophenyl carbonate, PEG-NPC) and was obtained from Sigma Chemical Company (St. Louis, MO, catalog number P9299). The average molecular weight of the PEG polymer was 3000±300 and is designated PEG-3000. Other PEG polymers that were used contained a succinimidyl propionate (SPA) leaving group, one with a single chain of PEG having a molecular weight of 20,000 (catalog number 2M4M0P01 from Shearwater Polymers, Inc., PEG-20,000), one having two chains of PEG, each with a molecular weight of 20,000 (PEG-(20,000)₂, product number 2Z3X0T01 from Shearwater Polymers, Inc.), and another having a molecular weight of 3400 (product number 4M4M0F02, Shearwater Polymers, Inc.).

Excess FFRck or FPRck (0.05 M) was mixed with PEG-3000 (0.02M) and allowed to react at room temperature in 100 mM HEPES buffer, pH 8.5, for 14 to 15 hours. In some experiments, 200 mM FPRck and 1 mM of amino-reactive PEG derivative was used. The product, PEG (3000)-FFRck, contained a PEG polymer

covalently linked to the amino terminus of FPRck. The product was separated from unreacted peptide by gel filtration on Sephadex G-25 and elution with Tris buffer. PEG-3000-FPRck eluted at the exclusion volume of the column, well separated from FPRck. During incubation, residual amino-reactive reagent was inactivated by water hydrolysis.

Reactions of H₂N-FPRck with PEG-20,000 and PEG(20,000)₂ were conducted in a similar manner but were complete within 2 hours at room temperature. Unreacted H₂N-FPRck was removed by dialysis of the reaction mixture against 0.05M Tris buffer, pH 7.5, for 48 to 72 hours. For both reactions, the amount of product formed was quantitated by UV absorbance of phenylalanine in the active site-directed inhibitor, using an extinction coefficient of 260 M⁻¹cm⁻¹. The concentration of PEG-FPRck also was estimated by reaction with human thrombin (Enzyme Research Labs. Inc.). Thrombin (50 μM) reacted quantitatively with PEG-FPRck reagents (<50 μM) within one hour at room temperature. Remaining thrombin was quantitated by its amidolytic activity toward S2288. The concentration of PEG-FPRck was determined by the amount of thrombin that was inhibited.

The PEG-3000 derivative (approximately 2:1 mol/mol of protein) was mixed with factor VIIa (0.2 to 0.5 mg/ml) and allowed to react for 2 hr at room temperature in buffer (pH 7.5) containing 5 mM calcium. For quantitative reaction with the larger PEG derivatives, reactions were allowed to proceed for 15 hours at room temperature.

Reaction with factor VIIa was monitored by loss of factor VIIa amidase activity toward the chromagenic substrate S-2288 (0.2 mM, Kabi) in a solution containing 100 nM soluble tissue factor (Dr. Walter Kiesel) and 5 mM calcium. When all factor VIIa activity was gone, the product was analyzed by standard SDS-PAGE methods. The sample was dialyzed to remove excess reagent.

Factor VIIa has a molecular weight of 50,000 and the expected molecular weight of the product was 53,000. On SDS-PAGE, however, PEG (3000)-VIIa migrated at a molecular weight greater than that of the bovine serum albumin standard (MW=67,000). SDS does not bind to the PEG portion of the molecule, which may account for the unusual migration in the gel. In addition, PEG is a very extended, unordered structure and may appear very large.

Reaction with long chain PEG-FPRck molecules was less vigorous than with PEG-3000. A typical preparation of VIIai(PEG-40kDa) is illustrated. Factor VIIa (0.51 mg in 0.9 mL of Tris buffer containing 5 mM calcium) was made 16.5 μ M in PEG-40,000-FPRck (a 1.5:1 ratio of reagent to protein). After 16 hours at room temperature, amidolytic activity, measured in buffer containing 100 nM soluble tissue factor and 0.36 mM S2288 substrate, was 12% of original. This preparation could be used directly for circulation turnover. Since unreacted factor VIIa has a circulation half-time of 35 minutes, most was removed before the first sample was taken at 90 minutes. At a ratio of PEG-40,000-FPRck to VIIa of 4.5:1, 97% of the amidase activity was inhibited after 15 hr at room temperature. In some cases, residual factor VIIa was removed by chromatography on Sephadex G-100. The derivative eluted at the exclusion volume of the column, well separated from free VIIa. PEG-modified protein was quantitated by absorbance at 280 nm, with WT-VIIa as the standard.

PEG (20,000)-VIIai migrated at an apparent molecular weight of 100,000, rather than at the expected molecular weight of 70,000. Again, the higher apparent molecular weight may stem from the PEG portion of the molecule.

When assayed by competition with factor VIIa (see below), the PEG-VIIai preparations had activity only slightly below that of the VIIai without PEG. Some reduction in activity was detected that correlated with the molecular weight of the PEG polymer. The PEG(3400)-Q10E32ai mutant had activity indistinguishable from the Q10E32ai protein. The PEG(20,000)-Q10E32ai and PEG(20,000)₂-Q10E32ai had activities approximately 40% that of the Q10E32ai molecule. This change may arise from a lower diffusion coefficient of the derivatized molecule. As the PEG polymer is very extended, it increases molecular volume much more than molecular weight. Diffusion coefficients, however, are proportional to molecular radius, while molecular volume is proportional to the particle radius cubed. Thus, a 2.5-fold decrease in diffusion can represent as much as a 6-fold increase in molecular volume. This decrease in function is small relative to the beneficial effect of PEG on circulation lifetime of these proteins.

Example 2 – Activity of PEG-modified Factor VIIai:

Activity was measured in two assays. Acute coagulation was assessed in a reaction initiated by adding tissue factor (TF) (37.5 μ L of 20 mM calcium containing 2.5 μ L of Innovin) to 112.5 μ L of Tris buffer containing plasma (37.5 μ L of factor VII-deficient plasma), VIIa (0.48 nM) and VIIai.

- 5 The assay showed little difference between WT-VIIai and Q10E32ai. The second assay was performed at equilibrium. Various amounts of factor VIIai, factor VIIa (20 nM) and TF (2.5 μ L of Innovin) were incubated for 1 hour in 112.5 μ L of Tris-BSA buffer containing 6.7 mM calcium. Factor VII-deficient human plasma (37.5 μ L) was added to start the reaction.

- 10 Data were plotted by the modified Hill equation (equation 1):

$$\log[\text{VIIai}] = \log[\text{VIIai} \cdot \text{TF}] / [\text{VIIa} \cdot \text{TF}] + \log(K_{\text{VIIai}} / K_{\text{VIIa}}) + \log[\text{VIIa}] \quad (\text{Eq. 1}).$$

- Concentrations of inactive complex (VIIai*TF) and active enzyme(VIIa*TF) were estimated from clotting times of reactions that did not contain inhibitor (CT_0) and reactions that did contain inhibitor (CT). Without inhibitor, the slope of $\log(\text{Clotting time})$ versus $\log[\text{VIIa}]$ was -0.30 so the relationship for solution of equation 1 is given by equation 2.

$$\text{Log}[\text{VIIai} \cdot \text{TF}] / [\text{VIIa} \cdot \text{TF}] = \log \{ (1 - 10^{(\log(\text{CT}_0/\text{CT})/0.3)}) / 10^{(\log(\text{CT}_0/\text{CT})/0.3)} \} \quad (\text{Eq. 2})$$

- Plots of $\log [\text{VIIai} \cdot \text{TF}] / [\text{VIIa} \cdot \text{TF}]$ versus $\log[\text{VIIai}]$ for two factor VIIai variants differed by the affinity of the respective proteins for TF. Reactions limited by the rate of factor VIIai- or factor VIIa- assembly with TF were analyzed by the same relationship. Linear graphs used all experimental determinations and represented the best fit analysis by the program Kaleidagraph.

- Three WT-VIIai(PEG) derivatives and three Q10E32ai(PEG) derivatives were assayed by the equilibrium assay using 4 titrations for each PEG type. The average ratio and standard deviation of WT-VIIai-PEG (or Q10E32ai-PEG) at 50% inhibition ($\log[\text{VIIai} \cdot \text{TF}] / [\text{VIIa} \cdot \text{TF}] = 0$) to that of WT-VIIai (or Q10E32ai) at 50% inhibition ($[\text{VIIai-PEG}]_{0.5} / [\text{VIIai}]_{0.5}$) for these titrations is shown (Figure 2) plotted as a function of the molecular weight of the PEG. The average, as well as all individual plots, showed concave downward shape (Figure 2, inset). This may correlate with a mechanism of PEG impact (see below).

Assay of WT-VIIai and Q10E32ai at equilibrium in the presence of competing factor VIIa showed about 25-fold higher function of the mutant. Function of VIIai(PEG-40,000) was reduced to about 25% that of WT-VIIai while function of Q10E32ai(PEG-40,000) was reduced to 40% that of Q10E32ai (Figure 3). Similar loss of function upon PEG modification indicated that the rate limiting steps were retained for both proteins. Overall, Q10E32ai(PEG-40,000) had 8-fold higher function than WT-VIIai without PEG.

Example 3 - Synthesis of bivalent inhibitor from DTPA anhydride: Active-site inhibitors were covalently linked to diethylenetriaminepentaacetic acid anhydride (DTPA), a bifunctional anhydride (Sigma Chemical Co., St. Louis, MO), to produce a bifunctional product. DTPA was slowly added as a solid to a solution of FFRck (0.04 M) at a pH 8.5 and reaction products were separated by HPLC. Samples were applied to a C18 column (Vydac) in reaction buffer. The column was washed with 0.1% trifluoroacetic acid (TFA), and a gradient of 20-24% acetonitrile in 0.1% TFA was applied from 4 to 24 minutes. A peak eluting at 20.8 minutes was identified by mass spectrometry as the desired bifunctional product DTPA-DiFFRck (molecular ion at 1358.2). A reaction product that eluted at 13.73 minutes was identified as the monovalent DTPA-FFRck product and had a monoisotopic mass of 876.

Example 4- Production of Vitamin K-dependent polypeptide homodimers:

Factors VIIai-VIIai, VIIai(Q10E32)-VIIai(Q10E32) and Xai-Xai homodimers were generated using DTPA-DiFFRck. One equivalent of DTPA-DiFFRck was added to each of the protein solutions (0.15±0.05 mg/mL) and allowed to react at room temperature and a pH of 7.5 in the presence of 5 mM calcium. Formation of product was monitored by loss of amidase activity as described in Example 1. In some cases, phospholipid vesicles (1.5:1.0 w/w vesicles/protein) were added to increase the rate of product formation. Binding of proteases to the membrane is thought to place their active sites in close proximity and enhance production of the dimer. When all enzyme activity was inhibited, the product was isolated by gel filtration chromatography on a column of either Sephadex G-100 or Sephacryl S-200HR. The dimer eluted before monomeric protein and was identified by SDS-PAGE. The apparent molecular weights were appropriate for the dimeric proteins.

Dimeric, PEG modified factor VIIai containing the mutations P10Q/K32E was produced using a PEG-derivatized, bifunctional active-site inhibition reagent. A bifunctional SPA derivative of PEG (product number 4M4M0F02, Shearwater Polymers, Inc.) having a PEG molecular weight of 3400 (5.7 mg) was added slowly, as a solid, to a solution of FFRck (8.3 mg in 0.125 mL of 0.1 M HEPES buffer, pH 8.5). The reaction proceeded for 15 hr at room temperature and the product was separated from unreacted FFRck by chromatography on Sephadex G-25.

The PEG-derivatized, active-site inhibition reagent eluted at the exclusion volume of the G-25 column and was quantitated by absorbance of phenylalanine at 260 nm, as above. Equimolar quantities of PEG-derivatized active site inhibition reagent and mutant factor VIIa (44 pmol in 0.115 mL of 0.05 M Tris buffer pH 7.5 containing 0.1 M NaCl and 5 mM CaCl₂) were mixed and allowed to react at room temperature for 4 hours. After 1.5 hr, 4 µg of phospholipid vesicles (phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine, 40/40/20) were added. Electrophoresis of the product showed approximately 30% yield of dimer with the rest of the protein migrating at the same position as PEG(3000)-VIIai. Analysis of activity of this mixed product by the competition assay described below showed that its inhibitor activity, on a weight basis, was equal to that of the monomeric VIIai mutant without PEG attached.

Example 5 - Heterodimers of VIIai-Xai and wild type Val-VIIai (Q10E32):

Factor VIIa was reacted with a 6-fold molar excess of bifunctional DPTA-DiFFRck reagent to prevent formation of a dimer of wild type VIIa. The product, VIIai-FFR-DPTA-FFRck, was separated from excess reagent by gel filtration on Sephadex G-25.

The product then was reacted with factor Xa (bovine protein) to form VIIai-FFR-DPTA-FFR-Xa, also referred to as VIIai-Xai. Product was separated from monomeric proteins by gel filtration on Sephadex G-100. The molecular weight of the VIIai-Xai heterodimer was appropriate for this species (MWs of factor Xa and factor VIIa are 46,000 and 50,000, respectively).

Heterodimers of VIIai-VIIai(P10Q/K32E) were formed without isolation of intermediates. An equimolar quantity of DPTA-DiFFRck was mixed with a solution of

factor VIIa (0.1 mg/mL) at room temperature and pH 7.5. Minimal amounts of dimeric VIIai, as estimated by SDS PAGE, were formed at these low protein concentrations. Equimolar amounts of mutant factor VIIa(P10QK32E) and phospholipid vesicles (1.5g lipid/g protein) were added to the reaction mixture. Factor VIIa and VIIa(Q10E32) both can bind to the vesicles, facilitating formation of crosslinks. Analysis by SDS-PAGE indicated that the yield of dimer was over 50%. High yields of dimeric VIIai (>50%) only were obtained in reactions that contained phospholipid vesicles. Dimers were separated from monomeric proteins by gel filtration on Sephadex G-100. Heterodimers of mutant VIIa(P10Q/K32E) with factor Xa were formed with similar reaction conditions, adding Xa in the second step.

Homodimers of mutant factor VIIa(P10Q/K32E) were made directly by addition of bifunctional DTPA-DiFFRck to a mixture of protein and phospholipid vesicles. Since this mutant binds tightly to the membrane, it was easily dimerized by the bifunctional active-site inhibition reagent. Homodimers of factor Xa were made by the same methods.

Example 6 - Superior activity of dimeric enzymes: A competition assay was used to assess the affinity of various proteins for membrane-associated tissue factor. Both VIIa and active-site modified factor VIIa bind to tissue factor. Factor VIIai prevents VIIa from binding and prevents generation of active VIIa-tissue factor complex, resulting in a loss of VIIa-tissue factor activity, which can be monitored by coagulation time in factor VII-deficient human plasma. Briefly, varying amounts of the VIIai derivatives were mixed with tissue factor-membrane (1 μ L of Innovin per 0.1125 mL of buffer-calcium solution containing 20 nM factor VIIa). Innovin (Dade Co.) is a commercial source of phospholipids and tissue factor. The reagents were allowed to equilibrate for 1 hr at 37°C then factor VII-deficient human plasma (0.0375 mL) was added and the time needed to form a clot was measured by the manual hand tilt method.

Figure 4A shows clotting time as a function of added VIIai-dimer and is compared with a similar titration with VIIai monomer. A large advantage of the dimeric protein is evident. To better compare these data, a Hill-type plot was created. Clotting times for various levels of VIIa-tissue factor, in the absence of VIIai, were determined and a

standard curve created. The clotting times in Figure 4A then were used in conjunction with the standard curve, to obtain the concentration of active VIIa-tissue factor complex in the solution. The level of inactive tissue factor-VIIai complex was then determined from equation 1:

5 Fraction of inactive complex = 1-fraction of active complex equation 1

The results were plotted as log (inactive/active tissue factor) vs. log VIIai concentration in Figure 4B. Two curves are shown for the dimeric protein. In one curve, the concentrations of all proteins were expressed on the basis of a 50kDa protein to compare the efficacy of dimer vs. monomer on a weight basis. The other curve used the molar concentration of dimer vs. the molar concentration of monomer. On a weight basis, dimeric VIIai had 16-fold higher activity than monomeric VIIai, while on a molar basis, the dimer was 32 times more active than the monomer.

The activity of the heterodimer, VIIai-Xai is shown in Figure 5. This heterodimer had 13-times higher activity than the monomer when compared on a weight basis. Thus, on a weight basis, the homodimer of wild type VIIai and the heterodimer of VIIai-Xai had similar potency. The homodimer of VIIai(P10QK33E) had the same activity as its corresponding monomer (Figure 6A). In the monomeric form, the Q10E32 mutant has much higher affinity than wild type factor VIIai due to an enhanced membrane binding site. Shah et al. (1998) Proc. Natl. Acad. Sci. USA, 95:4229-4234).

A heterodimer of mutant factor VIIai(P10Q/K32E) with wild type VIIai had the highest activity, 18-times that of monomeric wild type VIIai on a weight basis using titrations of the type and precision described above. This difference was on the basis of protein weight concentration. Surprisingly, the heterodimer of mutant VIIai(P10Q/K32E) with factor Xai had lower activity than the mutant monomeric protein (Figure 6B). Thus, dimer formation can have different impacts depending on the proteins used. Again, use of a different crosslinking agent, with altered linker arm, may allow this heterodimer to express higher activity. A homodimer of factor Xa also was an effective inhibitor of blood coagulation (Figure 7).

Example 7 – PEG-modified factor VIIa retains coagulation activity:

Factor VIIa (3.4 μ M, 170 μ g/mL) was derivatized with PEG₂-40,000-NHS (12mg/ml), PEG-10,000-SPA (17 mg/ml), or PEG-20,000-SPA at a 30:1 (reagent/protein) ratio in 75 mM HEPES buffer, pH 8.5, and at room temperature to produce factor VIIa with PEG randomly linked via lysine side chains. After reaction was complete (2 hours), the product was analyzed as described in Example 1. Amidolytic activity with soluble tissue factor was monitored and was 50 to 67% that of a control reaction. Coagulation activity was measured by a standard clotting assay in factor VII-deficient plasma. Calcium solution (37.5 μ L of 20 mM) was the final component added to a mixture of 37.5 μ L plasma, 1 μ L of Innovin, and 75 μ L of buffer. Clotting times were measured and compared to standards reactions with wild type factor VIIa. Coagulation activity of the derivatized protein was diminished relative to that of the control. Specific coagulation activity was 4% of WT-VIIa for VIIa-PEG-40,000 and 15% for VIIa-PEG-10,000. Thus, the coagulation function of VIIa-PEG appeared to be reduced by approximately the same amount that circulation lifetime would be increased. In the case of factor VIIa, PEG influences two steps, binding to TF and reaction with factor Xa. Despite little overall enhancement, factor VIIa with longer circulation lifetime may prove beneficial in some circumstances.

The reactions also were analyzed by SDS-PAGE, which indicated that almost all proteins had at least one PEG polymer attached. As expected from active site-directed derivatives, the mono-derivatized protein ran at an apparent molecular weight of 100,000. Other bands are detected at positions expected for two, three, and more PEG polymers per protein. This reaction showed that most of the enzyme activity survives one PEG polymer per enzyme.

High retention of activity was not obtained with activated protein C (APC), a protease that inhibits blood coagulation. That is, reaction with PEG and analysis of products by relevant blood clotting methods as well as SDS-PAGE showed that loss of APC activity approximately paralleled the formation of the mono-substituted protein. APC derivatized with a PEG-linked, active-site inhibitor may function as an inhibitor of prothrombinase.

Example 8 – Activity of PEG-modified Factor Xai and Factor IXai:

IXai-PEG and Xai-PEG were produced by methods similar to that for producing Factor VII-PEG. The PEG-modified proteins were separated from unmodified protein by gel filtration on Sephadex G-100. The PEG-40,000 proteins eluted at the exclusion volume of the column, well separated from free protein.

Factor Xai was examined by a diluted thromboplastin assay. In addition to factor Xai, the assay contained 112.5 μ L of standard Tris-BSA buffer with 6.7 mM calcium and 2.5 μ L of Innovin. Coagulation was initiated with 37.5 μ L of normal human plasma.

Factor IXai was assayed by an activated partial thromboplastin time (APTT). APTT reagent (50 μ L, Sigma) was mixed with normal human plasma (50 μ L) and incubated at 37°C for 3 minutes. Factor IXai and calcium solution (50 μ L of 20 mM) were added to start the reaction. Uninhibited assays gave clotting times of 29 \pm 2 sec.

The activity of Factor IXai-PEG and Xai-PEG was compared to activity of VIIai and PEG-VIIai. Factor VIIai-PEG showed a relatively small loss of function. In comparison, PEG-modified IXai and Xai lost all detectable activity. This contrast suggests a difference between the mechanisms of IXai/Xai action and VIIai action.

Example 9 - Circulation lifetime of proteins in the mouse: The mouse is an excellent experimental animal for study of factor VIIai turnover in the circulation. Murine tissue factor does not recognize human factor VII with high affinity and protein turnover can be studied without severe anticoagulation of the mouse.

Estimation of protein turnover in the mouse. Female BalbC mice (20 \pm 1 g Harlan) were warmed for 10 minutes by a heating pad placed under their cage to dilate the tail vein. They were anesthetized by intraperitoneal injection of Avertin (0.017 mL of a 1.25 % solution of tribromoethanol per g body weight). Factor VIIai (in 0.2 ml of buffer) was injected into the tail vein of the mouse at time zero then blood (20 μ L) was obtained from a small tail injury of the anesthetized mouse at various time points. The blood was anticoagulated with 0.1 M Na₃Citrate (9 parts blood to 1 of anticoagulant) and plasma was obtained after centrifugation of the cells. Factor VIIai then was assayed using equilibrium conditions, i.e., a competitive assay where VIIa and VIIai were allowed to reach equilibrium with available tissue factor before coagulation was started. The following procedure was developed to allow this condition without interference from

coagulation proteins in the mouse plasma. The mouse plasma was diluted 1:49 in 0.05 M Tris buffer, pH 7.5 (containing 0.1 M NaCl and BSA, 1 mg/mL), and Innovin (9 μ L/mL) and calcium (to 6.7 mM) were added to activate the clotting proteins in the diluted mouse plasma. After 2 hr at 39°C, the blood clotting proteases were inhibited by addition of diisopropylfluorophosphate (DIFP) to a final concentration of 2 mM. After 4 to 16 hours (e.g., 12 hours) at room temperature, the excess DIFP had spontaneously hydrolyzed and the solutions were assayed for inhibitor proteins. Fractions (0.28 to 5 μ L) of the diluted, activated and inhibited plasmas were added to 112.5 μ L of Tris buffer containing Innovin (1 μ L) and 30 pM factor VIIa in 6.7 mM calcium chloride- and BSA (1g/L)-containing buffer. After equilibration at 37°C for 30 minutes to 1 hr to form a complex between VIIai and tissue factor, normal human plasma (37.5 μ L) was added and clotting time recorded by the manual hand tilt method. The amount of inhibitor in the plasma was determined from this clotting time and by comparison to clotting times of reactions containing no inhibitor plasma but known amounts of factor VIIai. Samples without VIIai gave clotting times of 28 ± 2 sec. Precision was enhanced by dilution of all samples to give similar coagulation times (40 ± 5 s). Initial plasma factor VIIai concentration, immediately after injection, was about 600 nM. Background inhibition from control mouse plasma was usually not detected but always less than 5 nM.

Protein recovery in the circulation was high. Assuming 1.0 mL of plasma per animal, injected VIIai in the plasma should be 0.67 to 1.0 μ M. Recovery of VIIai at 5 minutes was the theoretical level. At 70-90 minutes, recovery was $42 \pm 14\%$ (n=7) of injected protein. PEG-modified proteins showed somewhat higher recovery, $54 \pm 11\%$ (n=4) of injected protein at 70-90 minutes. These recoveries were approximate as they assumed complete introduction of the protein into the bloodstream. Overall, the result showed high recovery for all injected materials with no unusual behaviors suggesting loss due to unknown factors.

First order decay plots are shown in Figures 8A and 8B. Linear analysis of the data provides an estimate of the rate constant for disappearance of VIIai. Initially, an injected protein undergoes protein turnover plus equilibrium into the extravascular space. Later, protein turnover is the only contributor to protein disappearance. The two mechanisms produced curvature in the first part of the first order decay curves (Figure

8A). This was avoided by measuring circulation halftimes starting at 70 to 90 minutes after injection.

Circulation halftimes were consistent. The average first order decay constant from independent analysis of the 5 mice receiving VIIai was -0.0074 ± 0.0027 (SD), corresponding to a circulation half-time of 93 ± 3 min. (Figure 8B). Modification by PEG-3400 gave a rate constant of -0.00345 ± 0.00005 (SD, $n=3$), corresponding to a circulation half-time of 201 min, 2.2-times longer than that of factor VIIai. Active site-modified PEG(20,000)-VIIai was removed from the circulation of three experimental animals with a rate constant $0.00081/\text{min} \pm 0.00003$, corresponding to a circulation half-life of 14 hours. This represents a 10-fold enhancement over normal VIIai. Factor VIIai(PEG-40,000) was removed from the circulation of two animals with a rate consistent of -0.00041 and -0.00044 , providing an average circulation half-time of 28.3 ± 2.3 (SD, $n=4$) hours, 18-fold longer than WT-VIIai. Given that larger species such as humans tend to have much longer protein circulation halftimes than the mouse, the PEG-derivatized polypeptides can be used to extend therapy in humans.

A summary of PEG derivatives is presented in Figure 9. Each derivative was administered to 2 to 5 animals. The derivative with PEG-80,000 represented the combined modification with PEG-40,000 at the active site plus PEG-40,000 attached by random modification of lysine side chains. While the extent of lysine modification was not determined, reaction was allowed to proceed to the point where activity was 50% that of the starting material (VIIai with PEG-40,000 at the active site). While some multiple modifications may exist, it was likely that the most active proteins were those with a single added PEG. Thus, a PEG mass of 80,000 was used for this preparation.

The circulation halftimes for the VIIai-PEG derivatives showed a striking, linear relationship with respect to molecular weight of PEG (Figure 10). The intercept occurred at 93 minutes, the value for VIIai without PEG. There did not appear to be an upper limit to circulation lifetime that could be achieved with PEG derivatives. This may conform to theoretical expectations, unless the mechanism of turnover becomes altered as PEG is increased.

The linear relationship was surprising and did not correlate with reports for Fv fragment of immunoglobulins, where an upper limit to circulation time was reported.

Thus, it appears that even longer circulation lifetimes are possible for the vitamin K-dependent proteins, provided that larger PEG polymers are attached.

Protein preparations injected into the mice often contained a slight excess of active site-directed reagent (PEG-FPRck). If the reagent modified endogenous
5 coagulation enzymes, it might create inhibitors that accumulate and could constitute a novel method of anticoagulation. However, control experiments showed that this did not occur. Two mice received four injections of PEG(20kDa)-FPRck (0.2 mL of 50 μ M) over a 3-day period. There was no apparent toxicity. When assayed for coagulation inhibitors by the VIIai assay described above, the plasmas showed normal background
10 inhibition. Consequently, all inhibitors detected in the assay arose from injected VIIai and its derivatives. Failure of PEG-FPRck to react with enzymes *in vivo* was expected since even dilute BSA (1 g/L) inhibited reaction as well. Initial levels of VIIai in the mouse plasma were 0.33-0.65 μ M, representing 50-75% of the theoretical level. The theoretical level was calculated from the assumptions that injection of the protein was
15 quantitative and that a 20 g animal has 1.0 mL of plasma. A small level of inhibitor was detected in control mouse plasma (0.01 ± 0.005 nM) and this was subtracted as a background.

Circulation of other materials is shown in Figure 11. All three animals given the VIIai proteins are shown in Figure 11A, along with PEG(3000)-VIIai and dimeric VIIai.
20 The dimer gave a rate constant for disappearance from the blood stream of -0.0052/min, corresponding to a circulation half-time of 139 minutes. Similar results were obtained with the factor VIIai-Xai heterodimer. Thus, a 3000 molecular weight PEG polymer was more effective than protein dimerization with respect to impact on circulation time. The impact of PEG cannot be explained by simple molecular weight change, since dimeric
25 factor VIIai has a molecular weight of 100,000 while PEG(3000)-VIIai has a molecular weight of 53,000.

Turnover of VIIai containing random PEG modification of surface lysines is shown in Figure 11B. Although the VIIa-PEG polymer mixture of Example 6 had activity that was a third less than that of the standard VIIai in the competitive assay
30 described in Figure 2, the loss of potency was more than offset by increases in circulation time of the derivative. Disappearance from the circulation was biphasic. The initial 50%

of the inhibitor activity was lost with a rate constant of $-0.00138/\text{min}$, corresponding to a circulation half-time of 9 hours. This may represent a combination of molecules with no PEG (circulation halftime of 94 min) and mono-derivatized factor VIIai (circulation half-time of 14 hr). The second half of the activity was lost with a rate constant of $-0.0004/\text{min}$. This corresponded to a circulation half-time of 29 hours, very similar to the rate of disappearance of active site modified PEG(20,000)₂-VIIai. It is possible that this half-time is for protein molecules with two PEG polymers. This suggests that the result in Figure 8B can be extended to addition of PEG to these proteins, by any mechanism. Thus, increases in circulation half-times appear to be based on molecular size or weight of the PEG polymer.

To test for the impact of PEG(20,000)-CO-FPRck administration, animals were injected four times (0.25 ml volume) with a total of 50 nmols of reagent into one animal over an 8 hr time period. Clotting assays did not detect an increase in coagulation inhibitors in this animal (assayed by the method outlined in example 8, above), indicating that little inhibition of blood coagulation proteases had occurred in those animals. If proteases were inhibited, prolonged circulation lifetime would cause them to accumulate and an increase of blood coagulation inhibitors would be observed. Over a period of 12 hours, there was no increase in the level of clotting inhibitors in this animal. Thus, PEG reagent containing the active site-directed group may not pose a toxicity problem to an animal. It is possible that excess PEG-modified active site reagent may not need to be removed from a reaction mixture before administration to an animal.

Example 10 - Analysis of factor VIIa function in whole blood of hemophilia patients: Heparin anticoagulation typically is monitored with an ACT (activated clotting time) assay that measured clotting time of whole blood. Commercial instruments are available for this assay; experiments herein used the Hemochron Jr. Signature microcoagulation instrument from International Technidyne Corp. The *ex vivo* response of blood from five hemophilia patients to added VIIa was assessed. In order to conduct this analysis with low stress to hemophilia patients, blood from normal individuals also was examined by treatment with anti-factor VIII antibody to generate an *ex vivo*

hemophilia A condition. Four normal individuals were tested in this manner. Representative results from both groups are shown in Figure 12.

Blood was drawn and the coagulation time immediately determined in the ACT. A second portion of the blood was mixed with citrate (9 volumes of blood with 1 volume of 0.1M Na₃Citrate) to prevent coagulation. Clotting time was assayed according to manufacturer's instructions for analysis of blood. Normal procedure uses fresh, non-anticoagulated blood. For experimental purposes, blood was anticoagulated with sodium citrate and then recalcified just before use. Recalcification was achieved by mixing one equivalent of calcium (2.5 μ L of 0.4 M CaCl₂ to 0.1 mL of anticoagulated blood) per equivalent of citrate used as the anticoagulant. This calcium was added just before loading the sample into the instrument. Blood that was anticoagulated and immediately recalcified gave clotting times that were 0-20% longer than the values obtained with the non-anticoagulated blood. This range was obtained for eight experiments performed on the four normal individuals. The range of clotting times for replicate measurements on each individual was about \pm 10%. Thus, anticoagulation and recalcification had a relatively small impact on clotting in the ACT. Most importantly, the clotting time for anticoagulated-recalcified blood was unchanged during storage at room temperature for 2 hr. The use of anticoagulated blood and its stability at room temperature allowed many samples to be run on one patient without the trauma of numerous blood withdrawals. Before each assay, the blood was mixed by gentle tipping of the plastic container.

Several types of cuvettes are supplied with the Hemochron Jr. instrument. The ACT+ cuvette contains an agent to activate the contact phase of coagulation as well as additional phospholipid to support later steps of the coagulation cascade. This cuvette was not useful for factor VIIa assays; all four hemophilia patients gave clotting times (126-138 s) that were in the range of normal individuals. A second type of cuvette, the ACT-LR, contains material (Celite) to activate the contact phase of coagulation but has no added phospholipid. This assay depends on cellular membranes to support the later steps of coagulation. In both cuvettes, the range of clotting times for normal individuals tested in this study was 100-180 seconds. In severe hemophilia, activation of the contact phase of coagulation is without impact since factor VIII and/or IX are entirely absent. The presence of Celite or Kaolin is, however, beneficial for evaluation of hemophilia

therapy as low levels of factor VIII and IX in some patients will contribute to coagulation. Thus, the ACT assay with the ACT-LR cuvette evaluates total coagulation potential from both the normal pathways and the high dose factor VIIa pathway.

Two hemophilia patients (I and II) had clotting times of >400 seconds in the ACT-LR cuvette. This is the upper limit of detection and the instrument simply shuts off. Normal individuals ranged from 126 seconds to 180 seconds. Two other hemophilia patients were described as moderately deficient. That is, they normally have >1% of the normal level of factor VIII or IX in their blood. One of these patients was factor VIII deficient (patient III) and the other patient was factor IX deficient (patient IV). Their moderate deficiency was reflected in the ACT times of 350 and 290 s, respectively. Patient I was assayed on two occasions with the second occasion occurring 10 days after receiving an infusion of factor VIII. Although performed at such a distant time, the impact of this therapy was detected with the ACT, which gave a clotting time of 308 s rather than >400 s without therapy (above). Thus, the ACT was capable of detecting even modest therapy levels.

Titration of hemophilia patients II and III with factor VIIa are shown in Figure 12A. Factor VIIa lowered coagulation time of the blood, as expected for a therapeutic agent. The relative clotting potential can be estimated by comparing these times to target levels for normal individuals undergoing heparin anticoagulation therapy (for dialysis, 250-300 s, for angioplasty and other catheterization procedures, 300-350 seconds, and for bypass surgery, >400 seconds per the manufacturer of the Hemochron Jr.). The current therapy with VIIa results in a level of about 50 nM. Figure 12A shows that this level of VIIa creates a coagulation potential similar to that of a normal patient who has been anticoagulated for angioplasty (300-350s, Figure 12A). This is a very modest coagulation potential. However, a modest potential is consistent with the need for repeated doses of VIIa administered over several hours (see above). Higher doses of VIIa resulted in decreased clotting time (Figure 12). Extrapolation indicated that dosage levels of >1000 nM would be needed to approach normal clotting times (180s).

Figure 12A shows that there was considerable patient variation with respect to response to VIIa. Patient III was about 2-fold more sensitive than patient II. Variability also was detected in the four normal individuals whose blood had been treated with anti-

factor VIII antibodies. Those four individuals also showed a 2-fold variation in response to factor VIIa. Thus, the ACT analysis appears to provide an indication of individual patient responsiveness to VIIa, information that is valuable in setting individual dosages during therapy.

Figure 12A also shows the responsiveness of patient II to the mutant factor VIIa containing the P10Q/K32E changes. This mutant has much higher affinity for the membrane and higher coagulation ability in tests such as those depicted in Figures 4 and 5. The greater potency of this mutant was readily detected in the ACT assay as well.

Titration of the second moderately severe hemophilia patient is not shown.

However, factor VIIa had a much smaller impact. For example, the 290 second clotting time of patient IV was reduced to only 240 seconds at 300 nM VIIa, a change of only 50 seconds. The patient who had received factor VIII infusion 10 days prior to analysis showed no detectable impact of factor VIIa until over 200 nM. A plateau of clotting time appeared to be reached at 240 seconds.

To create *ex vivo* hemophilia A (factor VIII deficiency), anticoagulated blood of four normal individuals was mixed with anti-factor VIII antibody (Enzyme Research Laboratories, Inc.). Sixty microliters of affinity purified antibody (protein absorbance at 280 nm=1.19) was mixed with 2 mL of blood. After 45 minutes at room temperature, the clotting time of all four individuals in the ACT-LR was greater than 400 minutes. Factor VIIa titrations for all artificial VIII-deficiency blood samples were carried out and an example is shown in Figure 12B. All individuals showed a response to factor VIIa with clotting times reduced to easily detected levels. Responsiveness was similar to that of the hemophilia patients, with a 2-fold range of response by the four individuals, as discussed above. The range was to a higher VIIa requirement. That is, two individuals required twice the VIIa to achieve the response shown in Figure 12B.

Response to VIIa was not predicted from other coagulation assays of the normal individuals, nor from their normal ACT clotting times. While all assays showed variation among the four normal individuals, shorter clotting time of two individuals in the classic APTT coagulation assay did not correlate with response to factor VIIa depicted in Figure 12. The normal ACT times (without anti-VIII antibodies) also did not correlate with responsiveness to factor VIIa. Thus, assay with factor VIIa in the procedure shown in

Figure 12 may provide a unique measurement for monitoring and designing individual factor VIIa therapies.

As for the hemophilia patients, the ACT-LR showed a different response to wild type vs. mutant factor VIIa (VIIa-P10Q/K32E). The difference was 4-fold for the individual shown in Figure 12B and ranged from 3.5 to 5-fold for the other three *ex vivo* factor VIII-deficient individuals. This was slightly less than the difference in the hemophilia patients. In any event, properties such as individual variation, protein specificity, detection of moderate vs. severe deficiency, and detection of therapy conducted after extended times suggest that the test shown in Figure 12 monitors important features of factor VIIa therapy in hemophilia.

Factor VIIa that had been modified by random reaction of PEG with lysines residues (see Example 7) also was tested. The molar concentration of active, modified VIIa was calculated from the amidolytic activity of the preparation versus that of a known concentration of unmodified factor VIIa. On this basis, activity of the PEG-modified protein was very similar to that of unmodified factor VIIa. The range of individual points for the PEG proteins (see Figure 12B) was greater than that that observed for normal VIIa. Greater variation for PEG proteins when assayed in the Hemochron Jr. signature microcoagulation analyzer was also detected during factor VIIa titration of blood from a hemophilia patient with factor VII deficiency.

Overall, limited PEG modification of factor VIIa by random derivatization of lysine residues can be used to generate an effective factor VIIa population.

Example 11— Acute versus Chronic Anticoagulation: Figure 13A shows anti coagulation by VIIai in an ‘acute’ situation. In this example, factors VIIa and VIIai are both present and calcium is added to initiate the coagulation reaction, mimicking the sudden exposure of tissue factor to the blood stream during injury. As indicated in Figure 13A, factor VIIai requires very high concentrations to inhibit the acute state. To compare the results of acute and chronic situations, factor VIIai concentrations in the acute reaction have been adjusted to recognize the concentrations VIIa (100 pM) in the acute experiment vs. that in the equilibrium experiment (5.6 nM). For comparison, results for factor Xai and activated protein C (wild type and a H10Q/S11G/Q32E/N33D mutant, data

from Nelsestuen et al. (1999) Trends. Cardiovasc. Med., 9:162-167) also are shown in Figure 13. Of these anticoagulants, factor VIIai is the least effective for acute state anticoagulation. APC is effective for treating acute anticoagulation, although it is rapidly inhibited in the blood and has a very short lifetime. Interestingly, the high affinity mutants are not very much better than wild type protein. Lack of difference can be explained by a reaction limited by diffusion of particles in solution. Association rate constants estimated for VIIa binding to tissue factor are $2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$, very near to the collisional rate constant. If acute anticoagulation is dependent on collisional rates, higher affinity will not have an impact. Only at equilibrium, when dissociation rates are also part of the interaction, will the high affinity mutants become effective.

Also shown in Figure 13A is the inhibition by factor VIIai under equilibrium or chronic conditions. At equilibrium, the higher efficacy and therefore the benefit of VIIai and its mutant P10Q/K32E become apparent. At equilibrium, 30 nM VIIai is sufficient to block a high level of coagulation (>90% inhibition). However, in acute anticoagulation, 30 nM VIIai is quite ineffective. It appears that VIIai is most desirable in anticoagulation of chronic situations while other inhibitors are more desirable for inhibition of acute situations.

The time course for conversion from acute to chronic inhibition is illustrated by the results in Figure 13B. Low levels of VIIai, insufficient to inhibit acute coagulation but creating high inhibition at equilibrium, were used. The time course for conversion to the inhibited state was determined by coagulation at various times after mixing. It is apparent that conversion to equilibrium conditions required 10 to 20 minutes. Thus, chronic inhibition by VIIai fits any situation where tissue factor may be exposed for this time or longer.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.

- 5 Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. An isolated vitamin K-dependent polypeptide linked to a polyethylene glycol (PEG) polymer.
2. The polypeptide of claim 1, wherein said polypeptide is selected from the group consisting of factor VII, factor II, protein C, protein S, gas6, and bone matrix Gla protein.
3. The polypeptide of claim 1, wherein said polypeptide and PEG polymer are directly linked.
4. The polypeptide of claim 1, wherein said polypeptide and PEG polymer are indirectly linked.
5. The polypeptide of claim 1, wherein said polypeptide is a protease selected from the group consisting of factor VIIa, factor IIa, and activated protein C.
6. The polypeptide of claim 5, wherein said protease is further linked to an active-site inhibition reagent.
7. The polypeptide of claim 6, wherein said active-site inhibition reagent is a chloromethylketone derivatized amino acid or peptide.
8. The polypeptide of claim 6, wherein said PEG polymer is linked to said protease via said active-site inhibition reagent.
9. The polypeptide of claim 5, wherein said protease is factor VIIa.
10. An active-site inhibition reagent linked to a PEG polymer.

11. The active-site inhibition reagent of claim 10, wherein said reagent is a chloromethylketone derivatized amino acid or peptide.
12. The active-site inhibition reagent of claim 10, wherein said reagent is a phosphohalide derivative.
13. An anticoagulant agent comprising two polypeptide monomers, wherein at least one of said polypeptide monomers is a vitamin K-dependent polypeptide, and wherein said polypeptide monomers are covalently linked.
14. The anticoagulant agent of claim 13, wherein said polypeptide monomers are covalently linked via a bi-functional active-site inhibition reagent.
15. The anticoagulant agent of claim 13, wherein said two polypeptide monomers are the same polypeptide.
16. The anticoagulant agent of claim 13, wherein said two polypeptide monomers are different polypeptides.
17. The anticoagulant agent of claim 13, wherein each of said two polypeptide monomers is a vitamin K-dependent polypeptide.
18. The anticoagulant agent of claim 17, wherein each of said vitamin K-dependent polypeptides is a factor VIIa polypeptide.
19. The anticoagulant agent of claim 14, wherein said bi-functional active-site inhibition reagent is linked to a PEG polymer.
20. The anticoagulant agent of claim 13, wherein at least one of said polypeptide monomers is directly linked to a PEG polymer.

21. A bifunctional active-site inhibition reagent comprising two covalently linked active-site inhibitors.

22. The bifunctional active-site inhibition reagent of claim 21, wherein at least one of said active-site inhibitors is linked to a PEG polymer.

23. A method of directly linking a vitamin K-dependent polypeptide to a PEG polymer, said method comprising incubating said PEG polymer with said vitamin K-dependent polypeptide for a time sufficient to link said PEG polymer to said vitamin K-dependent polypeptide, wherein said PEG polymer is reactive with amino groups or carbohydrate groups on said vitamin K-dependent polypeptide.

24. A method of indirectly linking a vitamin K-dependent polypeptide to a PEG polymer, said method comprising providing a PEG-modified, active-site inhibition reagent, wherein said PEG polymer is reactive with amino groups on said active-site inhibition reagent; and incubating said PEG-modified, active-site inhibition reagent with said vitamin K-dependent polypeptide for a time sufficient to link said PEG modified, active-site inhibition reagent to said vitamin K-dependent polypeptide.

25. A method of making an anticoagulant agent comprising incubating a bi-functional active-site inhibition reagent and at least one vitamin K-dependent polypeptide in the presence of phospholipid for a time sufficient to link said bi-functional active-site inhibition reagent and said vitamin K-dependent polypeptide.

26. A pharmaceutical composition comprising an isolated vitamin K-dependent polypeptide linked to a PEG polymer and a pharmaceutically acceptable carrier.

27. The pharmaceutical composition of claim 26, wherein said polypeptide and PEG polymer are indirectly linked.

28. The pharmaceutical composition of claim 26, wherein said polypeptide is a protease selected from the group consisting of factor VIIa, factor IIa, and activated protein C.

29. The pharmaceutical composition of claim 28, wherein said protease is further linked to an active-site inhibition reagent.

30. The pharmaceutical composition of claim 29, wherein said active-site inhibition reagent is a chloromethylketone derivatized amino acid or peptide.

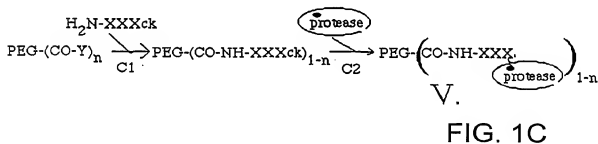
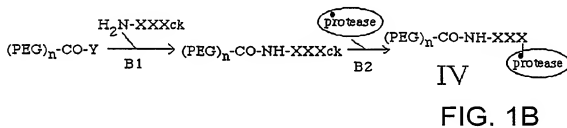
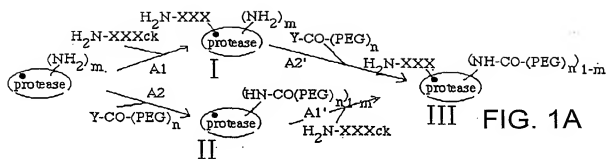
31. The pharmaceutical composition of claim 29, wherein said PEG polymer is linked to said protease via said active-site inhibition reagent.

32. The pharmaceutical composition of claim 28, wherein said protease is factor VIIa.

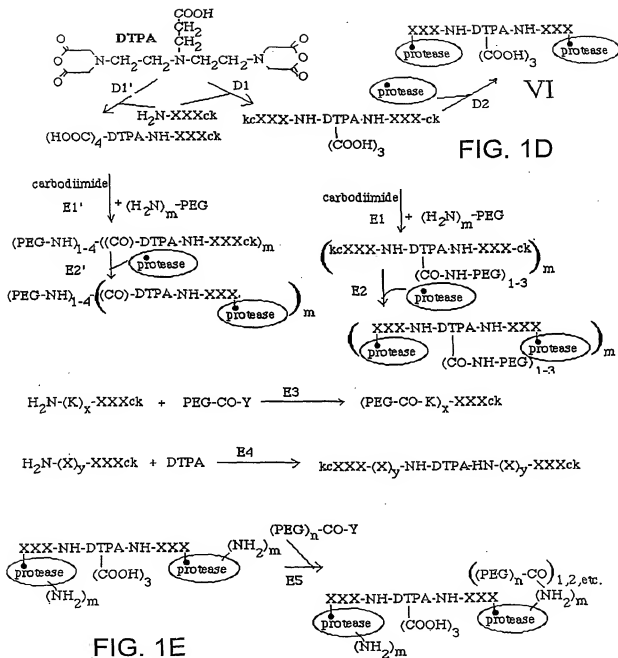
33. A pharmaceutical composition comprising an anticoagulant agent and a pharmaceutically acceptable carrier, said anticoagulant agent comprising two polypeptide monomers, wherein at least one of said polypeptide monomers is a vitamin K-dependent polypeptide, and wherein said polypeptide monomers are covalently linked.

34. The pharmaceutical composition of claim 33, wherein said polypeptide monomers are covalently linked via a bi-functional active-site inhibition reagent.

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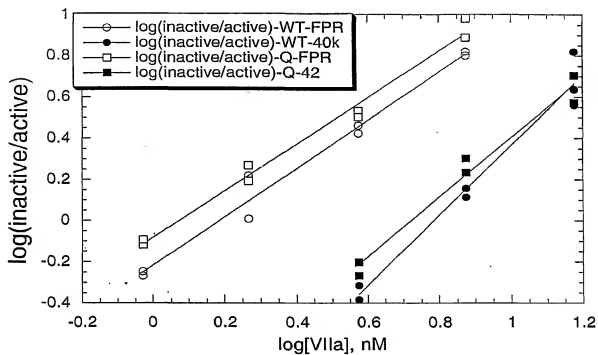
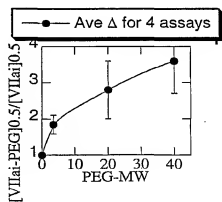


FIG. 2A



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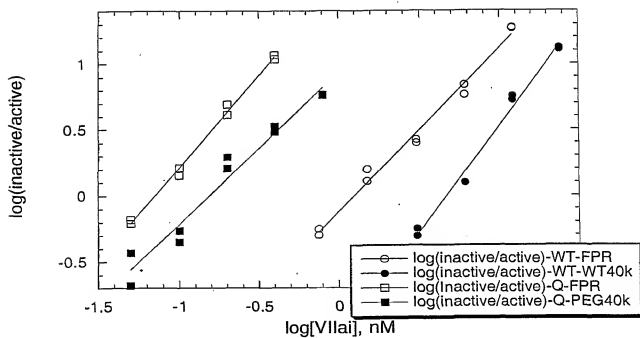


FIG. 3

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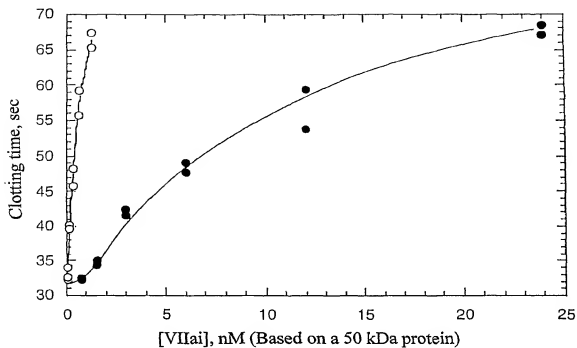


FIG. 4A

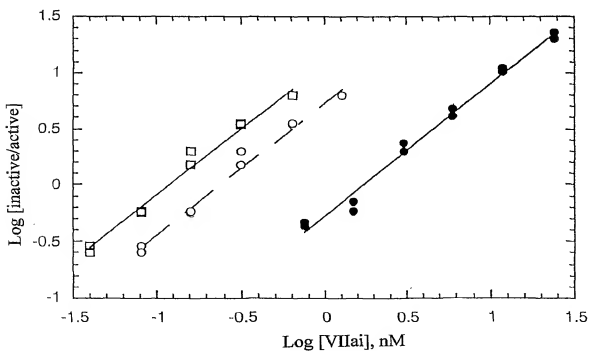


FIG. 4B

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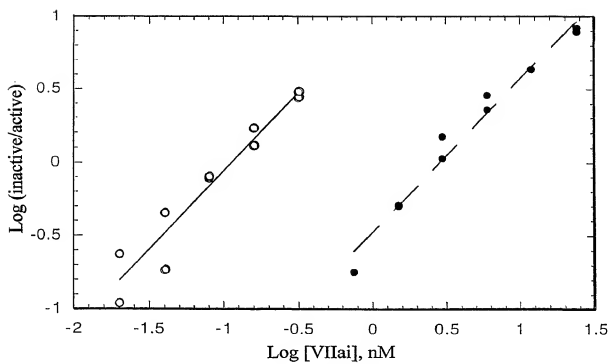


FIG. 5

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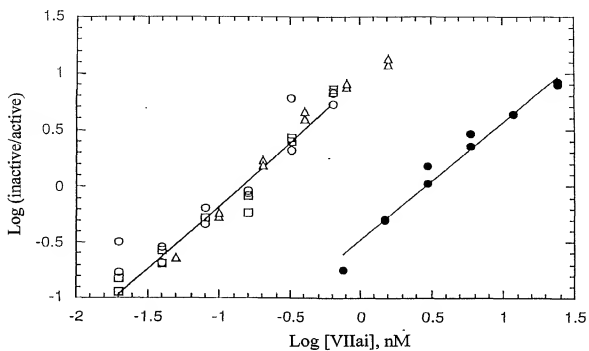


FIG. 6A

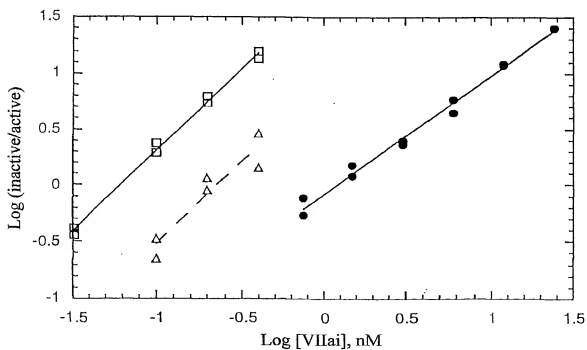


FIG. 6B

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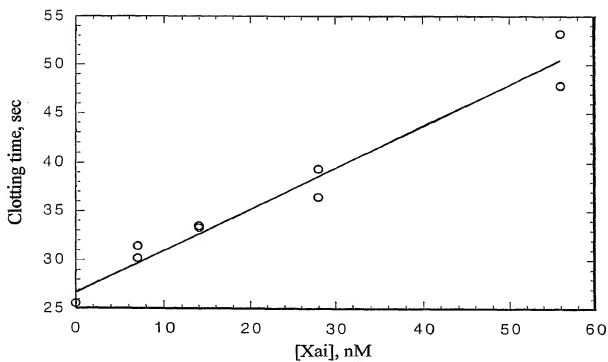


FIG. 7

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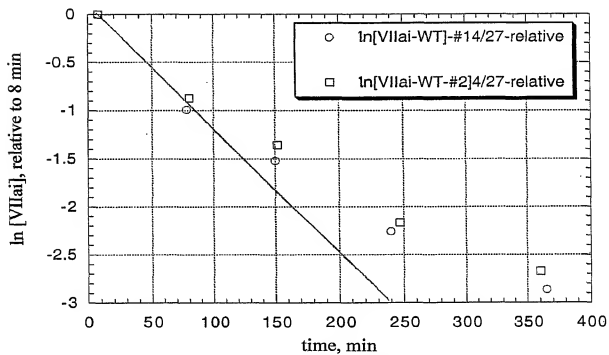


FIG. 8A

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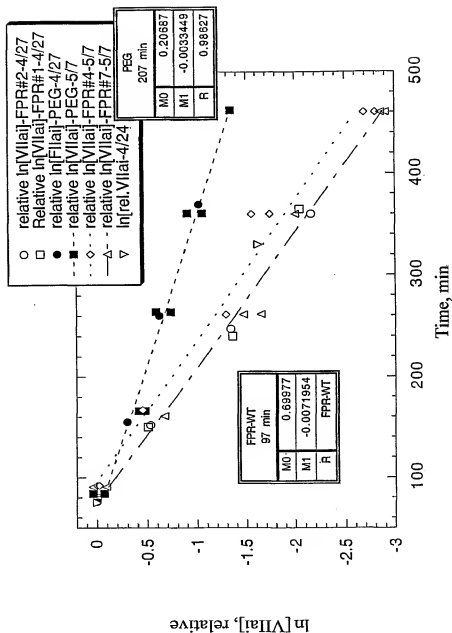


FIG. 8B

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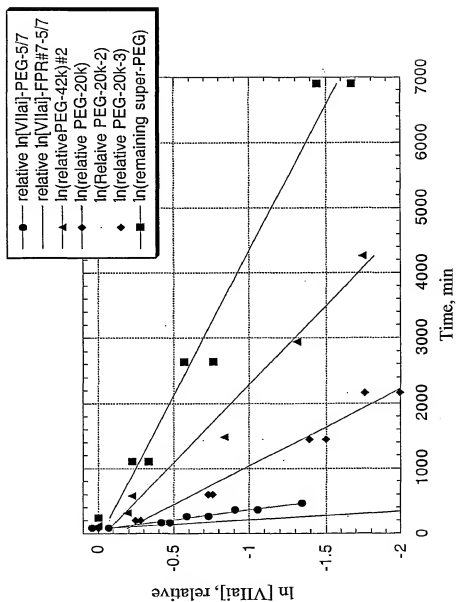


FIG. 9

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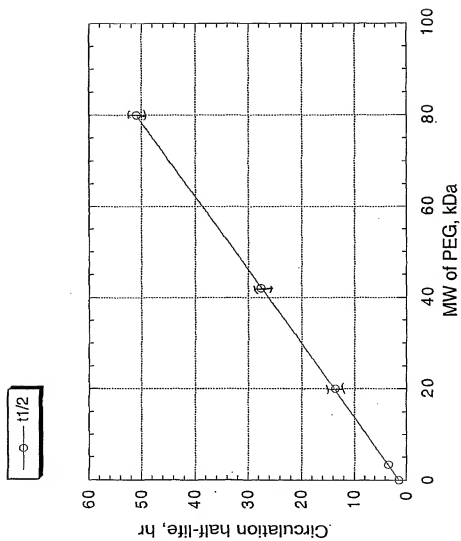


FIG. 10

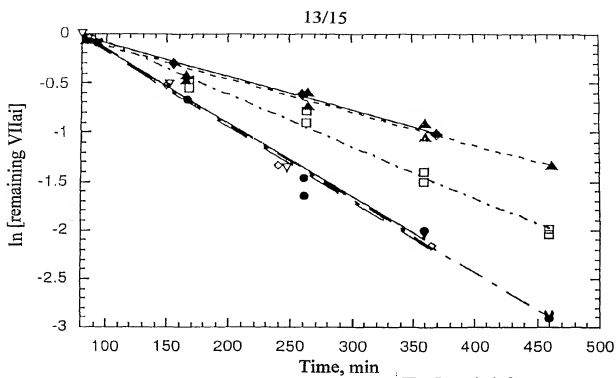


FIG. 11A

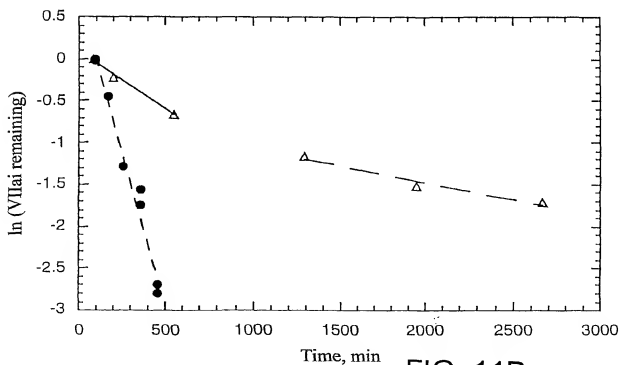


FIG. 11B

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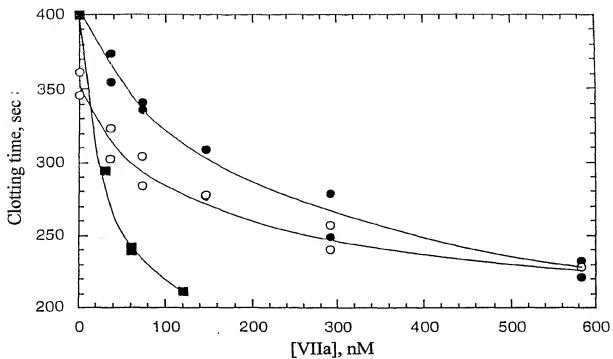


FIG. 12A

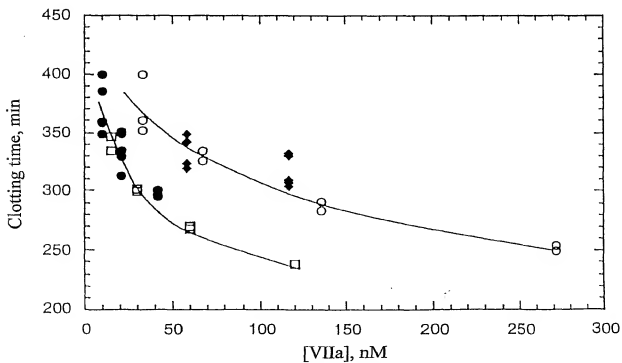


FIG. 12B

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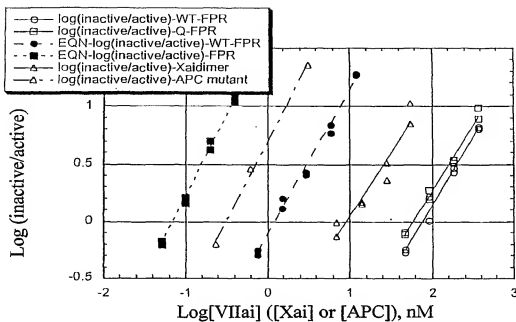


FIG. 13A

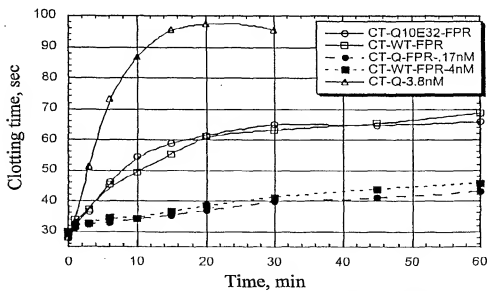


FIG. 13B